HIV-1: MOLECULAR BIOLOGY AND PATHOGENESIS

CLINICAL APPLICATIONS, 2ND EDITION

KUAN-TEH JEANG

ADVANCES IN PHARMACOLOGY

HIV-1: MOLECULAR BIOLOGY AND PATHOGENESIS

CLINICAL APPLICATIONS, Second Edition

HIV-1: MOLECULAR BIOLOGY AND PATHOGENESIS

CLINICAL APPLICATIONS, Second Edition

Edited by

Kuan-Teh Jeang

Molecular Virology Section,
LMM, NIAD, NIH
Bethesda, Maryland

ADVANCES IN
PHARMACOLOGY

VOLUME 56

AMSTERDAM • BOSTON • HEIDELBERG • LONDON
NEW YORK • OXFORD • PARIS • SAN DIEGO
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO
Academic Press is an imprint of Elsevier

Academic Press is an imprint of Elsevier
84 Theobald's Road, London WC1X 8RR, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands
Linacre House, Jordan Hill, Oxford OX2 8DP, UK
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1900, San Diego, CA 92101-4495, USA

First edition 2008

For information on all Academic Press publications
visit our website at books.elsevier.com

ISBN: 978-0-12-373601-7
ISSN: 1054-3589

PRINTED AND BOUND IN UNITED STATES OF AMERICA
08 09 10 11 12 10 9 8 7 6 5 4 3 2 1

Contents

Contributors xi
Preface written by Robin A. Weiss xv

Global Molecular Epidemiology of HIV: Understanding the Genesis of AIDS Pandemic

Yutaka Takebe, Rie Uenishi, and Xiaojie Li

I. Chapter Overview 1
II. Introduction 2
III. Genotype Classification of HIVs 4
IV. Global Distribution of HIV Genotypes 9
V. Methods for Identifying HIV Genetic Forms 11
VI. Origin of HIVs and Genesis of HIV-1 Pandemic 13
VII. Biological Significance of HIV-1 Variability and Recombination 14
VIII. Conclusions 18
References 18

Current Clinical Treatments of AIDS

Erin-Margaret Murphy, Humberto R. Jimenez, and Stephen M. Smith

I. Chapter Overview 27
II. HIV Medications 28
III. HIV Treatment 52
IV. Conclusion 63
References 63

HIV-1-Specific Immune Response

Alexandre Harari and Giuseppe Pantaleo

I. Introduction 75
II. Humoral HIV-1-Specific Response 77
III. HIV-1-Specific T-Cell Responses 78
References 87

Targeting HIV Attachment and Entry for Therapy

Julie Strizki

I. Chapter Overview 93
II. Background 94
III. Inhibition of Viral Attachment 95
IV. Chemokine Receptors in HIV Infection 98
V. Targeting Coreceptor Binding 99
VI. Fusion Inhibitors 107
VII. Resistance to Inhibitors of Viral Entry 109
VIII. Use Entry Inhibitors as Microbicides 110
References 111

Inhibitors of HIV-1 Reverse Transcriptase

Tatiana Ilina and Michael A. Parniak

I. Chapter Overview 121
II. Introduction 122
III. The Target 123
IV. Nucleoside RT Inhibitors 127
V. Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs) 140
VI. Other Inhibitors of HIV-1 RT 150
VII. Conclusion 154
References 154

Development of Protease Inhibitors and the Fight with Drug-Resistant HIV-1 Variants

Hiroaki Mitsuya, Kenji Maeda, Debananda Das, and Arun K. Ghosh

I. Chapter Overview 169
II. Introduction 170
III. Targeting Viral Protease 170
IV. The Role of PIs and Challenges in HAART 174
V. "Boosting": A Critical Modification of Clinical Efficacy of PIs 175
VI. Viral Resistance to PIs 176
VII. PIs with Activity Against Drug-Resistant HIV-1 182
VIII. Conclusions 189
References 191

HIV-1 Integrase Inhibitors: Update and Perspectives

Elena A. Semenova, Christophe Marchand, and Yves Pommier

I. Chapter Overview 199
II. Foreword 200
III. Integration: A Crucial Step in the HIV Life Cycle 200
IV. Approaches to Inhibit HIV Integration 210
V. Inhibitors in Clinical Trials 216
VI. Inhibitors in Preclinical Development 217
VII. Perspectives 218
References 219

Topical Microbicides: A Promising Approach for Controlling the AIDS Pandemic via Retroviral Zinc Finger Inhibitors

Jim A. Turpin, Marco L. Schito, Lisa M. Miller Jenkins, John K. Inman, and Ettore Appella

I. Chapter Overview 229
II. The AIDS Pandemic and the Rationale for a Microbicide 230
III. Topical Microbicides in Preclinical Development 232
IV. Characteristics of an Ideal Microbicide 237
V. The Retroviral Zinc Fingers of HIV-1 NCp7 as a Potential Microbicide Target 238
VI. Characteristics of the SAMT Chemotype 240
VII. Application of Thioesters for Microbicides 244
VIII. Target Specificity of the SAMT NCp7 Inhibitors 246
IX. Conclusions 249
X. Addendum 250
References 251

Viral Drug Resistance and Fitness

Miguel E. Quiñones-Mateu, Dawn M. Moore-Dudley, Oyebisi Jegede, Jan Weber, and Eric J. Arts

I. Chapter Overview 257
II. Introduction 258
References 283

Gene Therapy to Induce Cellular Resistance to HIV-1 Infection: Lessons from Clinical Trials

Mauro Giacca

I. Chapter Overview 297
II. Introduction: Do We Need HIV-1 Gene Therapy? 298
III. Gene Therapy of HIV Infection: Lessons from Early Clinical Trials 300
IV. Gene Therapy of HIV Infection: Current Developments 306
V. Gene Therapy for HIV Infection: Where Are We Heading? 314
References 317

Identification of Potential Drug Targets Using Genomics and Proteomics: A Systems Approach

Zachary A. Klase, Rachel Van Duyne, and Fatah Kashanchi

I. Chapter Overview 327
II. Introduction 328
III. Viral Targets 329
IV. Cellular/Viral Protein–Protein Interactions 331
V. Viral-Induced Cellular Alterations 343
VI. Other Approaches 351
VII. Conclusion 354
References 355

Rapid Disease Progression to AIDS due to *Simian immunodeficiency virus* Infection of Macaques: Host and Viral Factors

Que Dang and Vanessa M. Hirsch

I. Chapter Overview 369
II. Introduction 370
III. SIV Strain Diversity 371

IV. Biology of SIV 371
V. Pathogenesis of SIV in Macaques 373
VI. Unique Immunologic, Virological, and Pathological Features of Rapid Disease 377
VII. Host Factors That Influence Disease Progression 383
VIII. Role of Virus Genotype/Phenotype in Rapid Disease Progression 384
IX. Summary 388
References 389

Nonprimate Models of HIV-1 Infection and Pathogenesis

Viet Hoang, Elizabeth Withers-Ward, and David Camerini

I. Chapter Overview 399
II. Introduction 400
III. SCID-Hu Thy/Liv Mice 401
IV. The hu-PBL-SCID Mouse 405
V. NOD/LtSz-SCID Mice 407
VI. Rag2$^{-/-}$ γC$^{-/-}$ and NOD-SCID γC$^{-/-}$ Mice 408
VII. Humanized Immune Competent Mice and Rats 409
VIII. HIV-1 Tg Mice 410
IX. Rabbit Model of HIV Pathogenesis 412
X. Conclusion 413
References 413

Perspectives for a Protective HIV-1 Vaccine

Marco Schiavone, Ileana Quinto, and Giuseppe Scala

I. Chapter Overview 423
II. Introduction 424
III. Current Strategies in Developing an HIV-1 Vaccine 429
IV. Concluding Remarks 441
References 442

Molecular Mechanisms of HIV-1 Vertical Transmission and Pathogenesis in Infants

Nafees Ahmad

I. Chapter Overview 453
II. Introduction 454
III. Timing and Mechanism of HIV-1 Vertical Transmission 456

IV. Factors Associated with HIV-1 Vertical Transmission 458
V. HIV-1 Infection Diagnosis in Neonates and Infants 460
VI. Prevention of HIV-1 Vertical Transmission 460
VII. Characterization of HIV-1 Associated with Vertical Transmission 462
VIII. Chemokine Receptors and HIV-1 Vertical Transmission 464
IX. Molecular Properties of HIV-1 from Mother–Infant Pairs Associated with Vertical Transmission 465
X. Characterization of Functional Domains of HIV-1 Genes Associated with Vertical Transmission 470
XI. Properties of HIV-1 Associated with Lack of Vertical Transmission 478
XII. Analysis of Immunologically Relevant Mutations in HIV-1 Isolates Associated with Vertical Transmission 481
XIII. Mechanisms of HIV-1 Pathogenesis and Disease Progression in Infants 485
XIV. The Future 491
References 492

The Viral Etiology of AIDS-Associated Malignancies

Peter C. Angeletti, Luwen Zhang, and Charles Wood

I. Chapter Overview 509
II. Introduction 510
III. Kaposi's Sarcoma 512
IV. AIDS-Associated Lymphomas 520
V. AIDS-Associated NHL 524
VI. HPV-Associated Cancers 527
VII. HPV—The Causative Agent 529
VIII. Conclusions 538
References 539

Index 559
Contents of Previous Volumes 575
Color Plate Section at the end of the book

Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Nafees Ahmad (453) Department of Immunobiology, College of Medicine, University of Arizona, Tucson, Arizona 85724

Peter C. Angeletti (509) Nebraska Center for Virology, School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588

Ettore Appella (229) Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

Eric J. Arts (257) Division of Infectious Diseases, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106; Department of Molecular and Microbiology, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106; Center for AIDS Research, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106

David Camerini (399) Center for Virus Research, University of California, Irvine, California 92697; Center for Immunology, University of California, Irvine, California 92697; and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

Que Dang (369) Laboratory of Molecular Microbiology, NIAID, NIH 4 Center Drive Bethesda, Maryland 20892

Debananda Das (169) The Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

Arun K. Ghosh (169) Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907

Mauro Giacca (297) Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano 99, 34012 Trieste, Italy

Alexandre Harari (75) Laboratory of AIDS Immunopathogenesis, Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland

Vanessa M. Hirsch (369) Laboratory of Molecular Microbiology, NIAID, NIH 4 Center Drive Bethesda, Maryland 20892

Viet Hoang (399) Center for Virus Research, University of California, Irvine, California 92697; Center for Immunology, University of California, Irvine, California 92697; and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

Tatiana Ilina (121) Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

John K. Inman (229) Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Oyebisi Jegede (257) Department of Cellular and Molecular Biology, Kent State University, Kent, Ohio 44242

Humberto R. Jimenez (27) Saint Michael's Medical Center, Newark, New Jersey 07102; Ernest Mario School of Pharmacy, Piscataway, New Jersey 08854

Fatah Kashanchi (327) Department of Biochemistry, Medical Center, The George Washington University, Washington, DC 20037

Zachary A. Klase (327) Department of Biochemistry, Medical Center, The George Washington University, Washington, DC 20037

Xiaojie Li (1) Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Tokyo, Japan

Kenji Maeda (169) The Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

Christophe Marchand (199) Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892–4255

Erin-Margaret Murphy (27) Saint Michael's Medical Center, Newark, New Jersey 07102

Lisa M. Miller Jenkins (229) Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

Hiroaki Mitsuya (169) Department of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860–8556; The Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

Dawn M. Moore-Dudley (257) Department of Molecular and Microbiology, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106

Giuseppe Pantaleo (75) Laboratory of AIDS Immunopathogenesis, Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland

Michael A. Parniak (121) Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Yves Pommier (199) Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892–4255

Miguel E. Quiñones-Mateu (257) Center for AIDS Research, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106; Department of Cellular and Molecular Biology, Kent State University, Kent, Ohio 44242; HIV-1 Program, Diagnostic Hybrids, Athens, Ohio 45701

Ileana Quinto (423) Department of Clinical and Experimental Medicine, University of Catanzaro, Catanzaro, Italy

Giuseppe Scala (423) Department of Clinical and Experimental Medicine, University of Catanzaro, Catanzaro, Italy

Marco Schiavone (423) Department of Clinical and Experimental Medicine, University of Catanzaro, Catanzaro, Italy

Marco L. Schito (229) Henry M. Jackson Foundation for the Advancement of Military Medicine, Division of AIDS, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Elena A. Semenova (199) Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892–4255

Stephen M. Smith (27) The New Jersey Medical School, Newark, New Jersey 07103; Saint Michael's Medical Center, Newark, New Jersey 07102

Julie Strizki (93) Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Yutaka Takebe (1) Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Tokyo, Japan

Jim A. Turpin (229) Preventions Sciences Program, Division of AIDS, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Rie Uenishi (1) Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Tokyo, Japan

Rachel Van Duyne (327) Department of Biochemistry, Medical Center, The George Washington University, Washington, DC 20037

Jan Weber (257) HIV-1 Program, Diagnostic Hybrids, Athens, Ohio 45701

Robin A. Weiss (xv) Division of Infection and Immunity, University College London, London W1T 4JF, United Kingdom

Elizabeth Withers-Ward (399) Center for Virus Research, University of California, Irvine, California 92697; Center for Immunology, University of California, Irvine, California 92697; and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

Charles Wood (509) Nebraska Center for Virology, School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588

Luwen Zhang (509) Nebraska Center for Virology, School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588

Preface

This important volume illustrates through a series of authoritative chapters how a greater understanding of the molecular biology of the *human immunodeficiency virus 1* (HIV-1) can be translated into controlling and treating infection. Although the virus is relatively simple, containing only eight genes, its interaction with the human host is complex. In the majority of cases, infection leads to death from immunodeficiency, wasting, and neurological disease—the symptoms that we call acquired immune deficiency syndrome (AIDS). Thus, the mortality rate resulting from untreated HIV-1 infection is greater than that of Ebola or SARS, and there is no recovered immune state.

In contrast to human AIDS, the progenitor of HIV-1, SIV-cpz, is largely nonpathogenic in its natural host, the chimpanzee. Subtle changes in the HIV-1/SIVcpz genome, for example, in the *nef* gene, may have played a role in the evolution of its virulence, but the innate immune activation as opposed to control in chimpanzees appears to correlate with development of disease in humans (see "HIV-1-Specific Immune Response" by Harari and Pantaleo). At first sight it seems paradoxical that activation of immune responses eventually leads to immune collapse, but the activation is inappropriate and chronic. It is more of a wonder that progression to AIDS is not faster, which attests to the regenerative powers of the immune system.

AIDS was first recognized in 1981, and has emerged from being a concern among "high-risk" groups to representing a worldwide pandemic, which has already resulted in an estimated 25 million deaths. Moreover, some 40 million people are currently infected by HIV-1 (see "Global Epidemiology of HIV: Understanding the Genesis of AIDS Epidemic" by Yutaka Takebe), and antiviral treatment is not yet available to the majority. Given that the greatest proportion of infected persons have acquired HIV-1 sexually, much emphasis is placed by some political leaders on control through abstinence, being faithful and, reluctantly, use of condoms, the so-called ABC of AIDS

prevention. While behavioral restraint is indeed a most worthy aim, it does not appear to be any more efficacious than similar exhortations were when syphilis emerged to be pandemic 500 years ago. However, most of us are motivated to go beyond moralizing and hand-wringing to seek practical ways to impose a break upon the spread of HIV-1.

It is important to recognize the immense part that science applied to medicine has already contributed to public health measures for HIV and AIDS, as exemplified in this volume. Scientific epidemiology demonstrated the modes of transmission of the underlying cause of AIDS well before the culprit itself was identified. Within two years of the discovery of HIV-1 in 1983, screening of blood donations was set in place in all industrialized nations, greatly reducing iatrogenic HIV infection. Monotherapy with the first antiretroviral drug, Zidovudine, went into clinical trial in 1986, although it took another ten years before combination antiretroviral therapy (ART) showed long-term efficacy in controlling the course of infection. The introduction of ART had a huge impact: in the USA and other western countries, the mortality from HIV-1 infection dropped by 75% and has remained at this low level; hospital facilities for AIDS patients emptied as HIV-1 infection became a treatable, though not curable, out-patient condition (see "Current Clinical Treatments of AIDS" by Murphy *et al.*).

Those who deny that HIV-1 is the cause of AIDS, and those who question the validity and benefit of ART, should hang their heads in shame over the needless deaths that heeding their siren voices has incurred. Yet the economic and logistic challenge facing world health is to roll out ART to all who require it. Meanwhile, as HIV-1 gradually acquires multiple drug resistance (see "Viral Drug Resistance and Fitness" by Quiones-Mateu *et al.*), just as bacteria become resistant to antibiotics, we need novel drugs and novel targets in order to keep ahead of HIV. In this volume, the fourth to seventh chapters point the way to promising new approaches to therapy.

Certain HIV-1 inhibitors also show promise for prophylactic use in order to prevent infection of persons exposed to the virus. Their efficacy has already been proven in greatly reducing mother-to-child transmission. Other inhibitors may have a role in formulations of vaginal microbicides, which should be available to women for discrete use, among them are HIV entry inhibitors (see "Targeting HIV Attachment and Entry for Therapy" by Julie Strizki) and possibly viral zinc finger inhibitors (see "Topical Microbicides: A Promising Approach for Controlling the AIDS Pandemic via Retroviral Zinc Finger Inhibitors" by Turpin *et al.*).

Innovative genetic tools which have been developed across a range of molecular medicine targets are being applied to a better understanding and treatment of HIV/AIDS. Thus, genomic and proteomic technologies can aid HIV drug discovery (see "Identification of Potential Drug Targets Using Genomics and Proteomics: A Systems Approach" by Klase *et al.*) and provide insight into AIDS-associated tumors (see "The Viral Etiology of

AIDS-Associated Malignancies" by Angeletti *et al.*). Gene therapy (see "Gene Therapy to Induce Cellular Resistance to HIV-1 Infection: Lessons from Clinical Trials" by Mauro Giacca) can be considered for treatment, if bone marrow stem cells and CD4 T-cell precursors can be rendered resistant to HIV-1 infection by this means. Moreover, a welcome corollary of HIV research has been the development of lentivirus vectors based on the HIV-1 genome for gene therapy and research in other diseases. Thus, HIV and AIDS are not an island unto themselves; rather, they both benefit from and provide for advances in other fields of biomedicine.

The greatest scientific challenge facing AIDS researchers is to develop a vaccine that will protect us from HIV-1 infection. The failure to date to develop a safe and efficacious HIV vaccine is not for want of trying. Rather, there are some formidable obstacles, owing to the variability of the virus (see "Global Epidemiology of HIV: Understanding the Genesis of AIDS Epidemic" by Yutaka Takebe) and the complexity and incomplete immune responses to lentivirus infections (see "HIV-1-Specific Immune Response" by Harari and Pantaleo and "Perspectives for a Protective HIV-1 Vaccine" by Schiavone *et al.*). Animal models (see "Rapid Disease Progression to AIDS due to *Simian Immunodeficiency Virus* Infection of Macaques: Host and Viral Factors" by Dang and Hirsch and "Nonprimate Models of HIV-1 Infection and Pathogenesis" by Hoang *et al.*) are crucial to gain insight into immune correlates of protection, as well as properly designed clinical trials (see "Current Clinical Treatments of AIDS" by Murphy *et al.*). In the early days of HIV/AIDS research, there were disputes over the proportion of scarce resources that should be devoted to vaccines instead of therapies, but the research and development funds now available from both governmental and charitable sources has ended this dilemma. To be sure, we need rigorous peer review, value for money, and international coordination. However, the commitment to tackle AIDS will permit us not only to combat HIV-1 in those already infected but also to place the utmost effort in seeking to protect future generations from this major scourge of humankind.

Robin A. Weiss
University College London

Yutaka Takebe, Rie Uenishi, and Xiaojie Li

Laboratory of Molecular Virology and Epidemiology, AIDS Research Center,
National Institute of Infectious Diseases, Tokyo 162-8640, Tokyo, Japan

Global Molecular Epidemiology of HIV: Understanding the Genesis of AIDS Pandemic

I. Chapter Overview

Global dissemination of the *Human immunodeficiency virus* (HIV) represents a dramatic and deadly example of recent genome emergence and expansion. Since HIV-1 group M began its expansion in human population roughly 70 years ago (in early twentieth century), it has been diversifying rapidly, now comprising a number of different subtypes and

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56001-1

circulating recombinant forms (CRFs). Molecular epidemiological method has been useful tool to analyze the origin of HIVs and to track a course of global HIV dissemination. It could also provide the information critical to prevention and future vaccine strategies. In this chapter, we describe the classification and distribution of HIV genotypes and the biological and public health implications of genetic variability of this deadly pathogen.

II. Introduction

The HIV/AIDS pandemic continues to expand globally at a rate of 13,000 new infections everyday. The Joint United Nations Program on HIV/AIDS (UNAIDS) estimates that 40.3 (36.7–45.3) million individuals are living with HIV/AIDS, and about 25 million patients have already died (UNAIDS/WHO, 2005). A total of estimated 65 million individuals have been thus infected with HIV worldwide since the epidemic started a quarter century ago. In 2005 alone, there were 4.9 (4.3–6.6) million new HIV infections and 3.1 (2.8–3.6) million AIDS deaths (UNAIDS/WHO, 2005). This could be translated as that 9.3 new infections and 5.9 AIDS deaths occurred every minute (or a new infection every 6–7 sec and an AIDS death every 10 sec) worldwide. Figure 1 illustrates the magnitude of HIV/AIDS epidemic in different regions of the world.

Heterosexual transmission remains the dominant mode of transmission and accounts for ~85% of all HIV infections worldwide. Sub-Saharan Africa is an epicenter of the pandemic and continues to have high rates of new infections [3.2 (2.8–3.9) million per year]. It accounts for ~65% of new infections occurred worldwide in 2005 (Fig. 1). While HIV/AIDS epidemics came later in Asia, Asia is becoming the epicenter of second largest epidemic with ~1 million infections annually, accounting for 20% of new infections in the world (Fig. 1). Outside of Sub-Saharan Africa, one third of HIV infections are acquired through injecting drug use, most of which (an estimated 8.8 millions) are in Eastern Europe and central and Southeast Asia. The interplay between injecting drug use and unprotected sex fuels the epidemics in many countries in Asia (Fig. 1).

Molecular epidemiology has been a useful tool to analyze the origin of HIVs and to track a course of global HIV spread. The study areas include the distribution of HIV genotypes in different geographic areas, route of global and regional virus spread, molecular features of emerging epidemics and regional outbreaks, and specific association with different epidemiologic features, such as risk behaviors. Recent investigations also provide the new data on the role of recombination in the generation of HIV genetic diversity and the frequency of dual and superinfections. In this chapter, we overview the recent advances in the study of global molecular epidemiology of HIV and discuss its biological and public health implications.

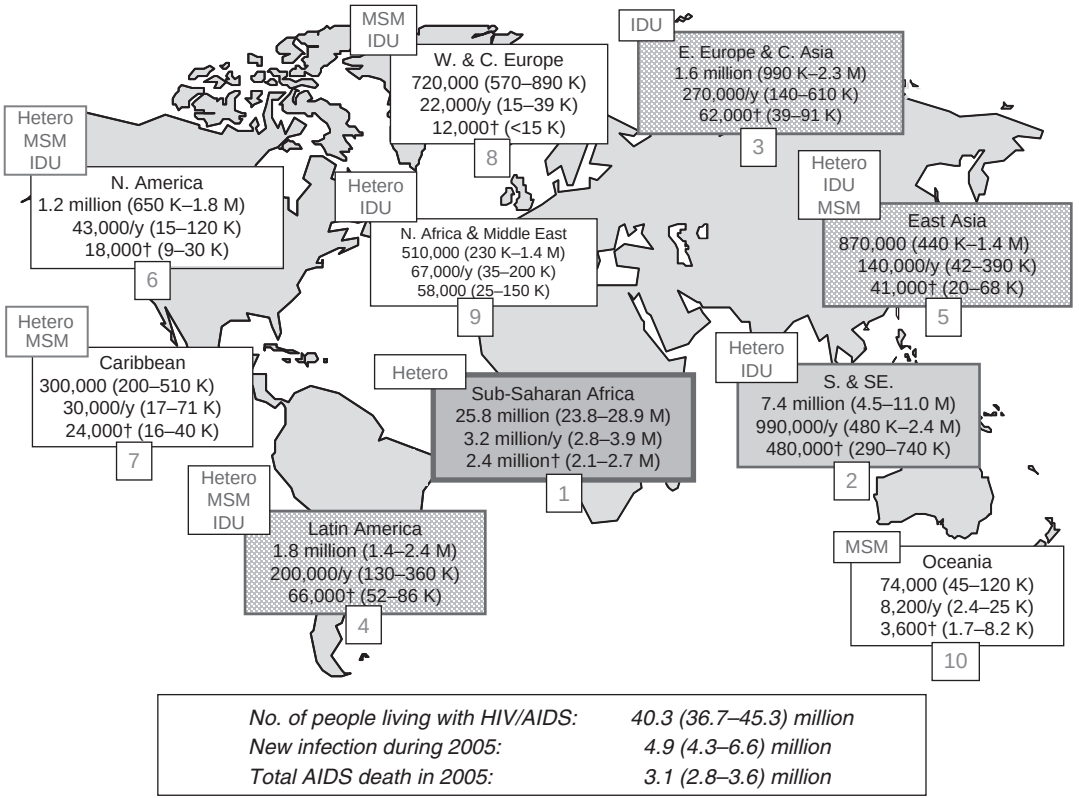


FIGURE 1 Distribution and estimated magnitude of HIV infections in different geographical regions of the world. UNAIDS/WHO estimates for the number of people living with HIV, people newly infected with HIV (/y) and AIDS death (†) in 2005 for the respective geographical regions are boxed. Predominant modes of transmission for each region are shown in the left upper box: Hetero, heterosexual; MSM, men who have sex with men; IDU, injecting drug user. Arabic numeral "1" in the box indicates the most afflicted region with the highest annual incidence and "10" indicates the least afflicted region. Global totals are shown at the bottom. Illustrations based on UNAIDS/WHO (2005).

III. Genotype Classification of HIVs

A. HIV Types (HIV-1 and HIV-2)

Etiologic agents for AIDS are subdivided into two related human retroviruses (lentiviruses): HIV-1 (HIV type 1) and HIV-2 (HIV type 2). HIV-1 is distributed worldwide, accounting for the majority of HIV infections. By contrast, HIV-2 is confined to West Africa and southern/western India (Schim van der Loeff *et al.*, 1999). Sporadic occurrences and transmission outbreaks of HIV-2 have been reported from many European countries (Cilla *et al.*, 2001; Damond *et al.*, 2004) and North and South America (Sullivan *et al.*, 1998) as well as Korea (Kim *et al.*, 2000; Nam *et al.*, 2006). It is known that the sexual and perinatal transmissions of HIV-2 are much less efficient than HIV-1 (Kanki *et al.*, 1994). This is attributed to a lower viral burden of HIV-2 during the relatively long asymptomatic period and may be the reason why the number of HIV-2-infected individuals has remained small, confined to limited geographic regions compared with HIV-1 infections (Schim van der Loeff *et al.*, 1999).

B. Genotype Classification of HIV-1s

Phylogenetic sequence analyses of HIV-1 strains distributed worldwide have identified three distinct groups of HIV-1 (M, N, and O), and nine genetic subtypes (A–D, F–H, J, and K) within major group (M) (Robertson *et al.*, 1999) (Fig. 2). The vast majority (more than 95%) of HIV-1 strains belong to group M (for Major or Main). Group O (for Outlier) comprises a pool of highly divergent, genetically related strains (Charneau *et al.*, 1994; Gurtler *et al.*, 1994; Loussert-Ajaka *et al.*, 1995; Vanden Haesevelde *et al.*, 1994) (Fig. 2). Group O infections are limited to people living in central Africa (Cameroon, Gabon, and equatorial Guinea), but even in this area they represent a small minority of HIV-1 infections. Only a few cases of group N (for New, or non-M/non-O) infections were identified in only limited number of patients from Cameroon (Simon *et al.*, 1998). HIV-1 group N infections fail to react serologically in standard whole-virus enzyme-linked immunosorbent assay (ELISA), yet are readily detectable by conventional Western blot analysis.

1. HIV-1 Subtypes

HIV-1 group M viruses are classified into at least nine discrete genetic subtypes (A–D, F–H, J, and K) based on the sequence of complete viral genomes (Fig. 2). In some subtypes, subclusters within subtypes were identified, leading to a classification into subsubtypes: The subtype F is subdivided into two subsubtypes: F1 and F2, and subsubtypes A1 and A2 strains were identified within subtype A (Fig. 2). The subtypes B and D are more

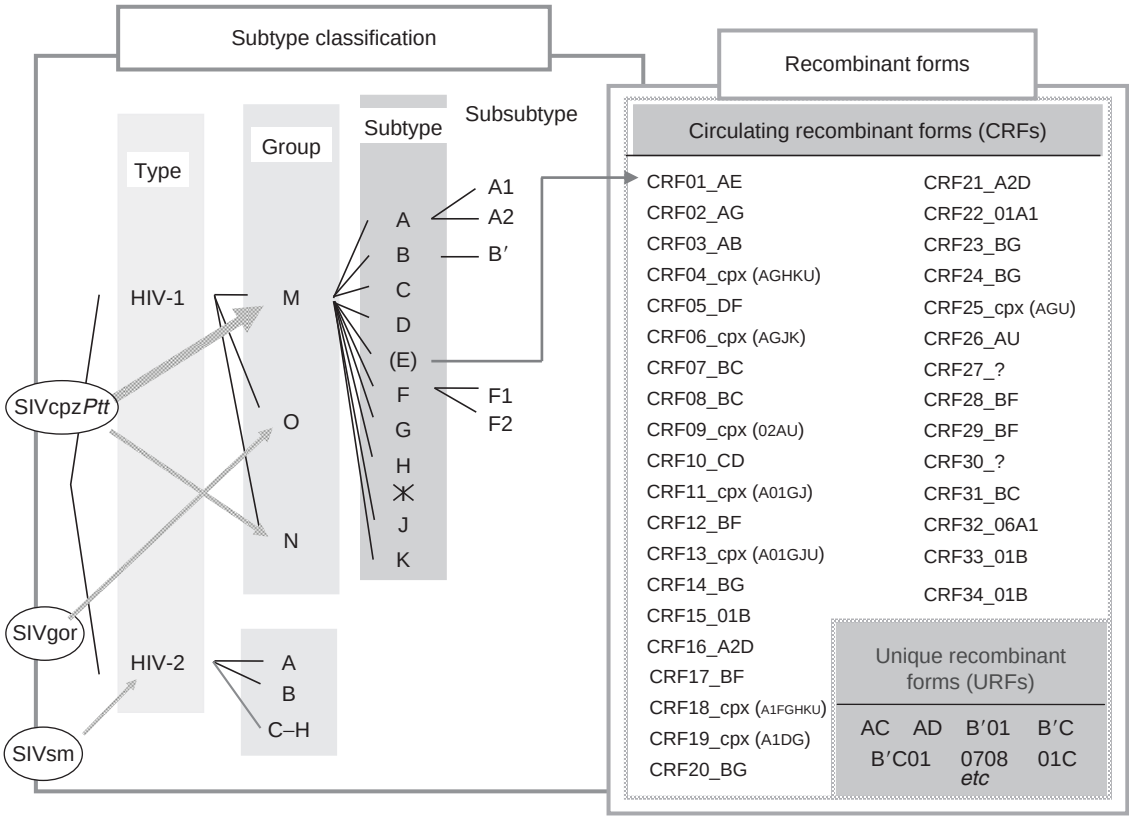


FIGURE 2 Classification of HIV genotypes and their origins. HIVs are classified based on the following four different strata: types (types 1 and 2), groups (M, O, and N for HIV-1; A–G for HIV-2), subtypes (A–K for HIV-1), and subsubtypes (A1 and A2, F1 and F2). HIV-1 recombinants in pandemic strains belonged to group M are categorized into circulating recombinant forms (CRFs) and unique recombinant forms (URFs) by their magnitude of dissemination. HIV-1 subtype B′ (B "prime") (Thailand variant of subtype B, also referred to as Thai-B), a unique regional subtype variant strongly associated with bloodborne transmission (prevalent among IDUs and former plasma donors/paid blood donors) in Southeast and East Asia. The plausible origins and routes of cross-species transmissions of HIVs are depicted in the left side (see text).

closely related to each other than to other subtypes, and therefore better considered as subsubtypes within a single subtype, rather than different subtypes. However, for the consistency with earlier published works, their original designation as subtypes is retained (Robertson *et al.*, 1999). Intrapatient genetic diversity of HIV-1 can vary from 6 to 10% in nucleotide sequence. HIV-1 isolates within a subtype may exhibit nucleotide distances of 15% in *gag* and up to 30% in *env* gp120-coding sequences. Intersubtype genetic diversity may range between 30 and 40%, depending on the gene analyzed. Similarly, the amino acid distances among different subtypes of HIV-1 group M reach ~25–30% in the *env* gene sequence and 15% in the *gag* gene sequence (Robertson *et al.*, 1999).

2. *HIV-1 Recombinants*

a. Circulating Recombinant Form It was realized that certain HIV-1 strains clustered with different subtypes in different regions of their genomes. Some of these mosaic HIV-1 genomes have been identified in several, apparently unlinked, individuals and play a major role in the global AIDS pandemic, designated as "CRFs" (Carr *et al.*, 1998). A total of 34 CRFs are currently recognized (http://hiv-web.lanl.gov/CRFs/CRFs.html) (Fig. 2). The global distribution of HIV genotypes are shown in Figs. 3 and 4. Under new nomenclature proposals, each CRF is designated by an identifying number, with letters indicating the subtypes involved. If the genome contains sequences originating more than two subtypes, the letters are replaced by "cpx," denoting "complex." To define a new subtype, subsubtype, or CRF, the representative strains must be identified in at least three epidemiologically unlinked individuals. Three near full-length genomic sequences are preferred, but two complete genome with partial sequences of a third strain are sufficient to designate a new subtype, subsubtype, or CRF (Robertson *et al.*, 1999).

b. Unique Recombinant Form In addition to CRFs, various types of "unique" recombinant forms (URFs) have been reported, currently without evidence of epidemic spread (McCutchan, 2000). URFs are diverse forms of HIV-1 intergenotype recombinants with unique mosaic structures, seen only in a single person or in a few epidemiologically linked individuals. Most of the URFs have been detected in the regions where multiple subtypes are cocirculating. A wide variety of URFs have been reported in the regions including Democratic Republic of Congo (DRC) (A/G/J and F1/K/U) (Vidal *et al.*, 2000), Tanzania (A1/C and A1/D) (Hoelscher *et al.*, 2001), Argentina (B/F) (Thomson *et al.*, 2000), Cuba (various combinations between subtypes A, B, D, G, and H) (Cuevas *et al.*, 2002), Spain (B/G) (Thomson *et al.*, 2001), India (A/C) (Lole *et al.*, 1999), Thailand (CRF01_AE/B) (McCutchan, 2000), Myanmar (various combinations between subtypes B′, C and CRF01_AE) (Motomura *et al.*, 2000, 2003; Takebe *et al.*, 2003), and China (B′/C) (Yang *et al.*, 2002, 2003 (Fig. 3). The detection of substantial

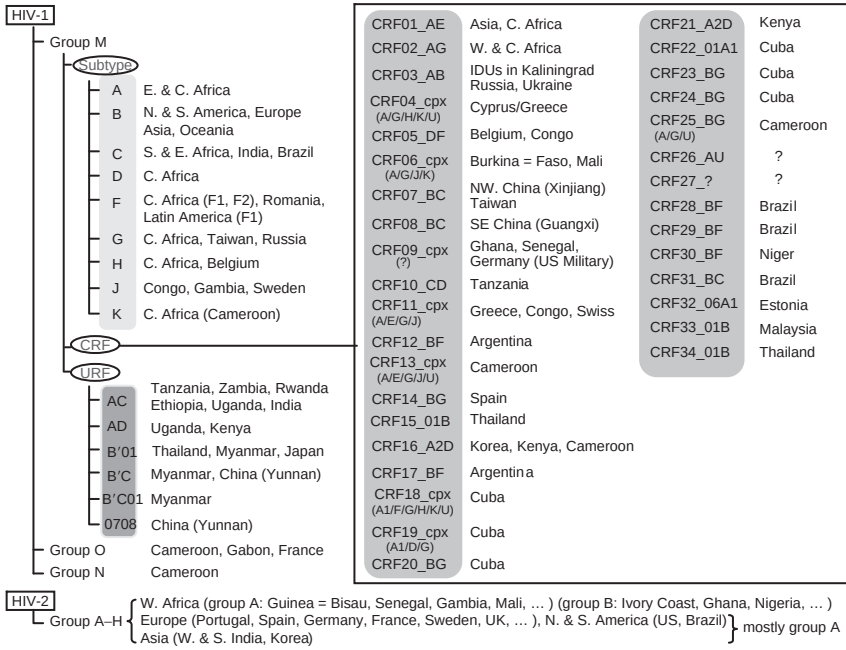


FIGURE 3 Geographical distribution of HIV genotypes. The global distributions of HIV-1 genotypes (groups, subtypes/subsubtypes, and CRFs/URFs) and HIV-2 groups are shown.

numbers of different URFs worldwide suggests that dual/multiple infections or superinfections with different lineages of HIV-1 strains might not be a rare event.

C. HIV-2: Genotype Classification and Geographic Distribution

HIV-2s are known to be members of a broader HIV-2/*Simian immunodeficiency virus* sooty mangabey (SIV-sm) phylogenetic group. HIV/SIV-sm phylogenetic groups are classified into eight genetic "groups" (A–H) (Damond *et al.*, 2004). Because these HIV-2 clades are nearly as distant from one another as are sequences from HIV-1 groups M, N, and O, HIV Nomenclature Committee decided to use "groups" rather than "subtypes" for HIV-2 genetic classification. Among these seven HIV-2 groups, only HIV-2 groups A and B are disseminated into significant numbers of human populations (Berry *et al.*, 2001; Schim van der Loeff *et al.*, 1999). HIV-2 group A has been identified predominantly in the western part of West Africa including Guinea, Bissau, Senegal, Gambia, and Mali. In contrast, HIV-2 subtype B has been found in central and eastern West African countries, including Ivory Coast, Ghana, and Nigeria.

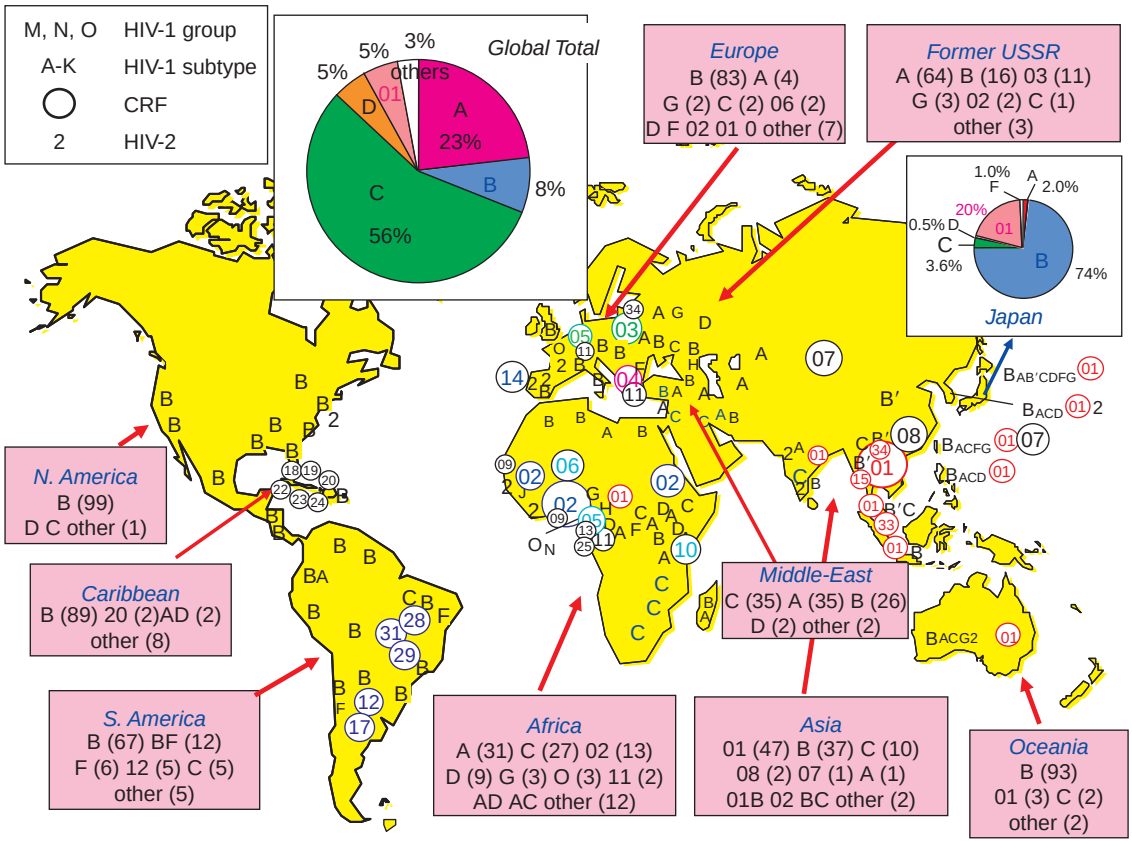


FIGURE 4 Global distribution of HIV genotypes and their estimated proportions. The number in parenthesis after each genotype is the proportion (in percentage) of the indicated genotype in the respective geographic regions. Data source (http://www.hiv.lanl.gov/components/hiv-web/new_geography/). A global total is adopted from Esparza and Bhamarapravati (2000). HIV-1 genotype distribution in Japan is also included.

Outside of Africa, unique HIV-2 transmission focuses and clusters were observed in India and Korea. HIV-2 infections were reported in West and South India, frequently associated with dual infections of subtype C strains (Grez *et al.*, 1994; Pfutzner *et al.*, 1992). Ten cases of HIV-2 infections have been reported so far in Korea, where two distinct clusters of HIV-2 (group A) infections were recognized (Nam *et al.*, 2006). The index case (seamen) responsible for each cluster appears to be infected in West Africa and subsequently transmitted the virus to its sexual partners through heterosexual and homosexual contacts inside Korea. While most of the HIV-2 infection cases in Asia are the infections with HIV-2 group A, one HIV-2 group B infection was identified in a person with Korean nationality living in Japan (Kusagawa *et al.*, 2003), who was likely to be infected through heterosexual contacts in DRC (Nam *et al.*, 2006).

IV. Global Distribution of HIV Genotypes

A. Global HIV-1 Variability

On a global scale, the most prevalent HIV-1 genotypes are subtypes C (56%), A (23%), B (8%), D (5%), and CRF01_AE (5%) in 1999 (Esparza and Bhamarapravati, 2000) (Fig. 4). The greatest genetic diversity of HIV-1 has been found in central Sub-Saharan Africa. Subtypes A and C are most common, but all groups and subtypes have been identified. The extensive diversification of HIV-1 group M appears to occur within or near the DRC, where the highest diversity of group M has been recorded (Kalish *et al.*, 2004; Vidal *et al.*, 2000), and the earliest case of HIV-1 infection, dating back to 1959 (Zhu *et al.*, 1998), has been documented. It is consistent to the notion that central Sub-Saharan Africa is the likely origin of current pandemic. In South and East Africa, subtype C predominates (Novitsky *et al.*, 1999) that causes the worst epidemic in those regions with the adult HIV prevalence of more than 30%. In West and West central Africa, the majority of circulating strains is CRF02_AG (Carr *et al.*, 1998). Subtype B viruses remain the most prevalent isolates in North and South America, Western and Central Europe, and Australia, and are also common in several countries in Asia (Hongkong, Japan, Korea, and Taiwan), northern Africa, the Middle East, and among South African and Russian homosexual men. In South America, subtype B is prevalent, while subtypes F and C, and CRF12_BF (Thomson *et al.*, 2000) and other B/F recombinants (CRF17, 28 and 29_BF) (Thomson *et al.*, 2002) have been reported (Section IV.C).

B. HIV-1 Variants in Asia

Studies revealed unique profiles of HIV-1 genotype distribution in Asia. HIV-1 subtype C predominates in India (Lole *et al.*, 1999), with estimated

5.7 million (3.4–9.4 million) people infected. HIV-1 CRF01_AE of central African ancestry is widely circulating in Southeast Asia (Ou *et al.*, 1992, 1993; Weniger and Brown, 1996). CRF01_AE epidemic broke out in the late 1980s among female commercial workers and their clients in Thailand and became prevalent among injecting drug users (IDUs) in this country (Ou *et al.*, 1993) and disseminated to neighboring countries in Asia, including Cambodia, Vietnam, Malyasia, China, Taiwan, Korea, and Japan (Weniger and Brown, 1996; Weniger *et al.*, 1994). Subtype B′ (Thailand variant of subtype B; also referred to as Thai-B virus) (Kalish *et al.*, 1995; Ou *et al.*, 1992, 1993) is a unique subtype B variant that spreads primarily through IDU networks in Southeast Asia (Motomura *et al.*, 2003; Weniger *et al.*, 1994). Two closely related CRFs, CRF07_BC and CRF08_BC, are disseminating rapidly among IDUs in northwestern (Xinjiang Province) (Su *et al.*, 2000) and southeastern (Guangxi Province) China (Piyasirisilp *et al.*, 2000), respectively. A variety of novel CRFs composed of CRF01_AE and subtype B (B′) in Thailand [CRF15_01B (Tovanabutra *et al.*, 2003) and CRF34_01B] and in Malaysia (CRF33_01B) have been identified (Tee *et al.*, 2006).

C. Other HIV-1 Variants of Geographical Relevance

Less prevalent HIV-1 subtypes, but common on a localized scale, are observed in various geographic areas: subtype D, distributed mainly in East Africa (Uganda, Tanzania, and Kenya); subtype F (subsubtype F1) predominant in Romania (mostly children infected through contaminated blood products and unsterilized needle and syringes), and found in a minority of HIV-1-infected people in Brazil; subtype G circulating in West and central Africa, with the highest prevalence in Nigeria as well as in Portugal and northern Spain. A variety of novel CRFs were identified in South America: CRF12_BF (Thomson *et al.*, 2000) and CRF17_BF in Argentina; CRF28_BF, CRF29_BF, and CRF31_BC in Brazil. These new recombinant strains account for ~12% of HIV-1 infections in Latin America (Fig. 4). In Europe, CRF14_BG and its related recombinants are circulating locally in northwestern Spain (Delgado *et al.*, 2002; Thomson *et al.*, 2001) and Portugal (Esteves *et al.*, 2002). The widest range of novel CRFs, including CRF18_cpx (Thomson *et al.*, 2005), CRF19_cpx (Casado *et al.*, 2005), CRF20_BG, CRF22_01A1, CRF23_BG, and CRF24_BG (http://hiv-web.lanl.gov/CRFs/), have been reported in Cuba, where those recombinants account for ~20% of HIV-1 infections (http://www.hiv.lanl.gov/components/hiv-web/new_geography/). Injecting drug use triggered a new HIV-1 epidemic in Eastern Europe: CRF03_AB was originally identified among IDUs in the Russian city of Kaliningrad (Liitsola *et al.*, 1998), and later detected in several cities in Ukraine and Belarus (St. Petersburg, Smolensk, and Perm) (Figs. 3 and 4). Other minor nonrecombinant subtypes (A2, F2, H, J, and K) were detected in central Africa. Most of the remaining CRFs have lesser relevance in epidemic on a global scale.

D. Emergence of HIV-1 Recombinants Worldwide

Although the exact prevalence of recombinant strains is not well known, the preliminary data show that the proportions of discordant *gag*/*env* samples varied from less than 10% to up to 40% in Africa (McCutchan *et al.*, 1999; Vidal *et al.*, 2000) and 10–30% in some areas in Asia, including central Myanmar (Kusagawa *et al.*, 1998; Motomura *et al.*, 2000, 2003; Takebe *et al.*, 2003) and more than 60% in western part of Yunnan Province (southwestern China) (Yang *et al.*, 2002).

Recombinant viruses have already contributed substantially to the global pandemic, and the likelihood of generating recombinant viruses will continue to increase as the different HIV-1 subtypes spread worldwide (Peeters, 2000). Mixing of different lineages of HIV-1 strains could quickly lead to the evolution of new recombinant strains. Even recombinant viruses will recombine, leading to the evolution of second-generation recombinants, inter-CRF recombinants (ICRs): ICR01_0708 identified among IDUs in Yunnan Province of China is the first example of this category, which is composed of two closely related CRFs in China, CRF07_BC and CRF08_BC (Yang *et al.*, 2003) (Fig. 3).

V. Methods for Identifying HIV Genetic Forms

A. Phylogenetic Sequence Analysis

For subtype classification, phylogenetic sequence analysis is the most reliable method. Various software programs for phylogenetic analysis (i.e., molecular evolutionary genetic analysis, MEGA) are freely available (http://hiv-web.lanl.gov/). Due to the high frequency of recombination in HIV, it is necessary to equip software programs designed for identifying recombination. This is particularly the case for the molecular epidemiological investigation in the areas where different lineages of HIV-1 strains are cocirculating. Softwares designed for detecting recombination, including Simplot (Ray, 2002) and Recombination Identification Program (RIP) (http://hiv-web.lanl.gov/) are useful for this purpose. Figure 5 illustrates an example of phylogenetic tree analysis and recombination breakpoint analyses (bootscanning plot and subregion tree analyses) for identification and characterization of novel recombinant strains in Myanmar (Takebe *et al.*, 2003).

B. Alternative Methods (Heteroduplex Mobility Assay and Serotyping)

Other methods, less expensive and requiring less sophisticated equipments, can be useful as the alternatives for sequencing. This includes serotyping and heteroduplex mobility assay (HMA) (Delwart *et al.*, 1993).

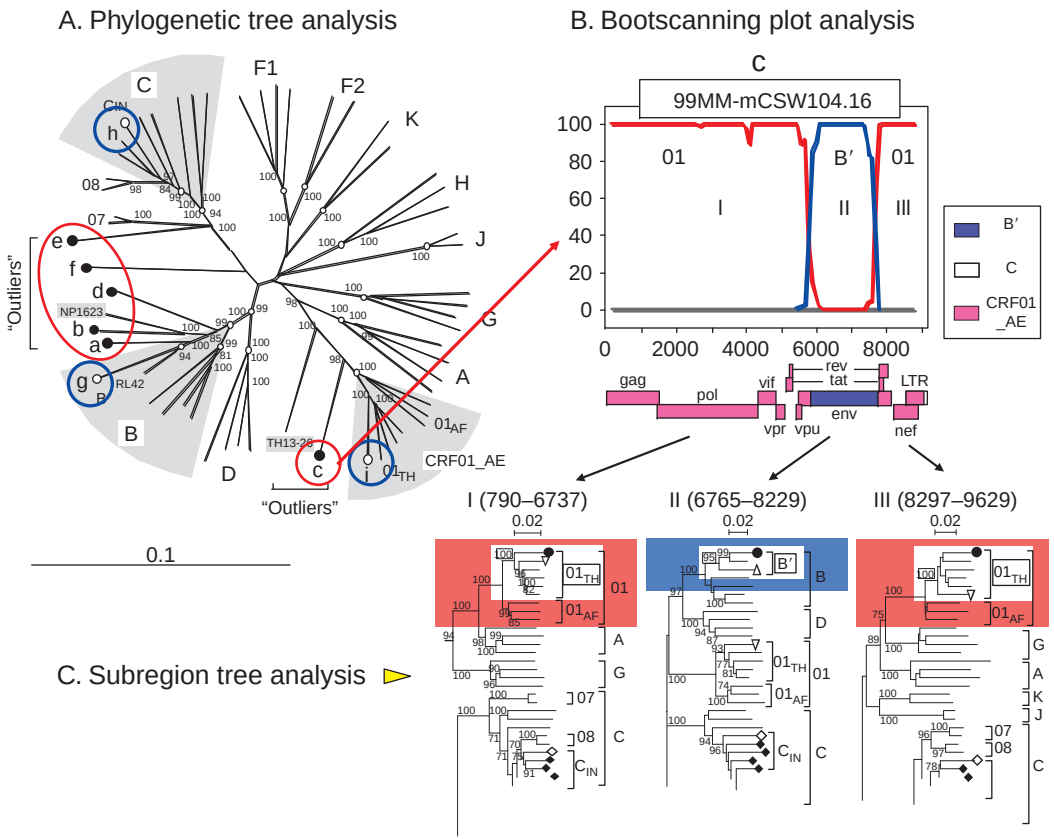


FIGURE 5 Phylogenetic tree analysis (A) and recombination breakpoint analysis for identification novel HIV-1 recombinant strains. Neighbor-joining tree analysis of Myanmar HIV-1 isolates based on near full-length sequences. Strains (g–i) belong to nonrecombinant forms of HIV-1 subtypes B′

Serotyping is a method based on the binding of antibodies present in the patient's sera to the peptides representing a segment of envelop V3 loop of different subtypes (Pau *et al.*, 1993, 1994). Serotyping is particularly useful for analyzing large numbers of specimens for epidemiological studies. However, this assay cannot reliably distinguish between subtypes A and C and cannot detect recombinants. HMA is the method based on electrophoretic mobility of DNA duplexes formed by hybridization of the polymerase chain reaction (PCR)-amplified sequences with reference sequences of different subtypes. However, both methods are less reliable in the areas with high HIV-1 genetic heterogeneity, such as central Africa and some regions in Asia, because of the high frequency of intermediate or incorrect results. These two methods can be useful in the areas, where one or two relatively homogeneous subtypes are prevalent. For instance, serotyping method was successfully applied for the distinction of subtype B and CRF01_AE infections for the study in Thailand (Pau *et al.*, 1993).

VI. Origin of HIVs and Genesis of HIV-1 Pandemic

A. HIV/AIDS as a "Zoonosis"

Current evidence indicates that HIV-1 and HIV-2 entered into human population through multiple zoonotic infections from SIVs-infected nonhuman primates (Hahn *et al.*, 2000). It has been known that HIV-2 and SIV sm have a high degree of genetic and phenotypic homology (Gao *et al.*, 1992), sharing unique open-reading frame, called vpx, in their genomes. Moreover, the habitat of the sooty mangabey closely matches HIV-2 endemicity in West Africa. These close relationships between HIV-2 and SIV-sm led to the hypothesis that HIV-2 infection is a zoonosis (Sharp *et al.*, 1999).

By contrast, HIV-1 is most closely related to SIV cpz isolated from the chimpanzee subspecies *Pantroglodytes troglodytes* (*P.t.t.*) (Corbet *et al.*, 2000; Gao *et al.*, 1999; Hahn *et al.*, 2000; Peeters *et al.*, 1997). The most diverse forms of HIV-1 are found in the geographic region corresponding to the range of *P.t.t.* in West equatorial Africa (Charneau *et al.*, 1994; De Leys *et al.*, 1990; Gurtler, 1996; Simon *et al.*, 1998), and HIV-1 groups and

(Thailand variant of subtype B) and C, and CRF01_AE, respectively. Strains (a–f) are "outliers" that are not assigned to any known HIV-1 genotypes (subtypes/CRFs). They turned out to be novel HIV-1 intergenotype recombinants, that is, "unique recombinant forms" (URFs). The data outputs obtained from various recombination breakpoint analyses, including bootscanning plot analysis (B) and subregion tree analysis (C) for "outlier" strain (c) are schematically illustrated. The results indicate that the strain (c) is a novel HIV-1 URF composed of subtype B′ and CRF01_AE of Thailand origins. The strain (c) shows the structural similarity to CRF15_01B, but is not exactly identical (Takebe *et al.*, 2003).

SIV cpz sequences are interspersed in phylogenetic trees, suggesting that there are shared viral lineages in human and chimpanzees (Corbet *et al.*, 2000; Gao *et al.*, 1999; Hahn *et al.*, 2000; Peeters *et al.*, 1997). Each group of HIV-1 and HIV-2 is believed to represent a distinct cross-species transmission of the viruses from its chimpanzee and sooty mangabey reservoirs, respectively (Hahn *et al.*, 2000). However, genetic survey of SIVs in African primate species in central Africa using fecal specimens identified HIV-1 group O-like viruses in wild gorilla (*Gorilla gorilla gorilla, G.g.g.*) (Van Heuverswyn *et al.*, 2006). Collectively, it could be speculated that SIV-cpz*Ptt* have crossed at least twice in humans, resulting in the AIDS pandemic by HIV-1 group M in one instance and infection of a few individuals in Cameroon by group N in another, and that the third HIV-1 lineage group O appears to be evolved from a virus from wild gorilla (*G.g.g*), while this virus (SIV gor) forms monophyletic lineages within SIV-cpz*Ptt* radiation (Sharp *et al.*, 2005). The plausible origins and routes of cross-species transmissions of HIVs are illustrated in the left side of Fig. 2.

B. Dating the Origin of Pandemic HIV-1 Strains

Korber *et al.* (2000) estimated the date of the most recent common ancestor (MRCA) of HIV-1 group M to be 1931 [95% confidence interval (CI): 1915–1941], suggesting that HIV-1 group M began its expansion in human population roughly 70 years ago. The phylogenetic analyses assuming molecular clock suggested that the founder of subtype B in the United States originated in 1967 (95% CI: 1960–1971). Similarly, the MRCA of CRF01_AE in Thailand was dated 1986 (95% CI: 1978–1989) (Korber *et al.*, 2000). By contrast, according to Lemey *et al.* (2003), the date of the MRCA of HIV-2 group A strains was estimated to be 1940 $\pm$ 16, and that of HIV-2 group B strains was estimated to be 1945 $\pm$ 14 in Guinea, Bissau. Taken together, zoonotic transfers of HIVs occurred in early or the first-half of the twentieth century and subsequently spread globally, generating the pandemic observed today. The origins and plausible route of dissemination of HIV-1 strains responsible for epidemic in Asia is schematically illustrated in Fig. 6.

VII. Biological Significance of HIV-1 Variability and Recombination

A. HIV-1 Subtypes and Disease Progression

It has been suggested that HIV-1 subtypes could influence viral transmissibility and pathogenecity. However, the existence of many other factors makes it difficult to establish the true effect of viral subtypes. A study in Thailand showed that the disease progression in the patients infected with

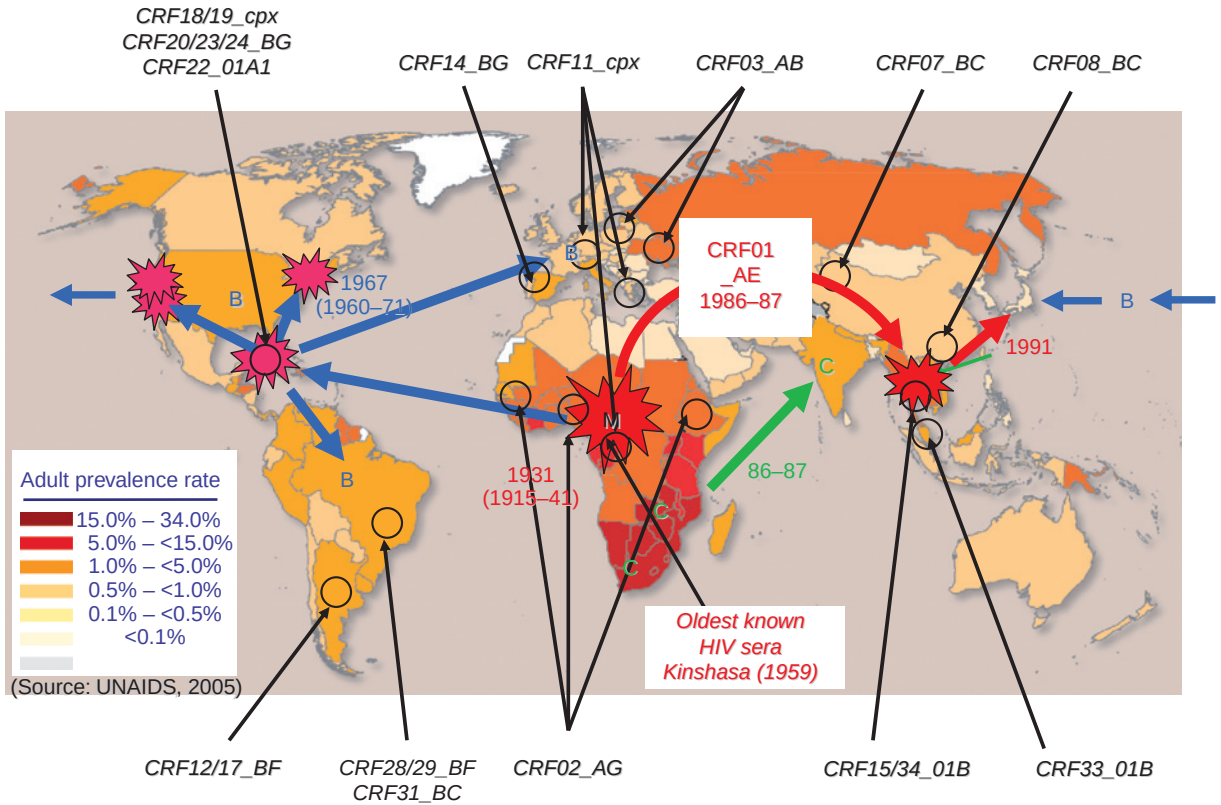


FIGURE 6 Origin of HIV-1 group M and plausible routes of the spread of the HIV-1 strains responsible for epidemic in Asia. The epidemic focuses of selected CRFs of geographical relevance are shown. The illustrations are superimposed on the world map with estimated adult HIV prevalence in different countries (UNAIDS/WHO, 2005).

CRF01_AE is similar to those observed in subtype B-infected populations in the West (Amornkul *et al.*, 1999; Kilmarx *et al.*, 2000).

In contrast, several studies showed that HIV-1 subtypes differ in rates of progression to AIDS: (1) A prospective study of female prostitutes in Senegal showed that women infected with subtypes C, D, or G were eightfold more likely to develop AIDS than subtype A (Kanki *et al.*, 1999); (2) The cohort study in Kenya, where subtypes A, C, and D cocirculate, plasma RNA levels were found to be highest in subtype C (Neilson *et al.*, 1999); (3) A study in Tanzania suggests that subtypes A and C and recombinant viruses are more likely to be transmitted perinatally than subtype D (Renjifo *et al.*, 2001), suggesting that maternal subtype may play a role in vertical transmission; (4) The response to the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) is increased in subtype C long terminal repeat (LTR) with nuclear factor κB (NFκB) configuration, suggesting that subtype C may have a replication advantage in individuals with chronic immune activation (Montano *et al.*, 2000); (5) A matched case-control study showed that viruses containing subtype C LTRs were six times more likely to be transmitted than those with subtype D (Blackard *et al.*, 2001). The study in Uganda showed subtype D was associated with faster progression to death and with a lower CD4 cell count than subtype A (Kaleebu *et al.*, 2002).

In contrast, a study from Sweden showed no differences in disease progression in subtypes A, B, C, or D (Alaeus *et al.*, 1999). However, it is not clear whether such differences are due to the environmental factors such as the prevalence of other infectious diseases that may induce the systemic "immune activation," including sexually transmitted diseases (STDs) and parasitic diseases. Indeed, several studies suggest faster disease progression in the persons infected in Africa, compared with those infected in the United States or Western Europe (Galai *et al.*, 1997; Kanki *et al.*, 1999). Long-term prospective studies in recent seroconverters will be needed to elucidate the relationship between HIV-1 genotypes and clinical disease progression.

B. HIV-1 Dual Infection, Superinfection, and Recombination

1. Dual Infection: Mechanism and Prevalence

Dual infections are the prerequisite for the generation of recombinants. When a single cell that is infected with genetically distinct viruses produces progeny virions with RNAs from each virus, recombination could occur between the two copackaged heterologous RNAs through strand switching during the next replication cycle (Malim and Emerman, 2001). Therefore, dual infection with more than one lineage of HIV-1 strains within an individual is a source of rapid viral evolution by recombination. Over the last decade, a number of cases of dual infections with the same or different HIV-1 subtypes through various transmission routes, including vertical

transmission (Janini *et al.*, 1998; Mellquist *et al.*, 1999), sexual transmission (Jost *et al.*, 2002; Zhu *et al.*, 1995), and blood transfusion or injection drug use (Diaz *et al.*, 1995; Ramos *et al.*, 2002; Sala *et al.*, 1994, 1995) have been documented.

2. Distinction Between Coinfection and Superinfection

By the temporal mode of the acquisitions of different HIV-1 strains, dual infections are divided into two categories: coinfection (simultaneous) and superinfection (sequential). Coinfection is defined as an infection with two heterologous strains ether simultaneously or within a brief period of time (arbitrarily within the first month of infection) before infection with the first strain has been established and an immune response has developed. In contrast, superinfection is defined as an infection with a second strain after the immune response to the first infection has been established (Smith *et al.*, 2005). As of August 2005, 16 published cases of superinfections have been reported worldwide (Smith *et al.*, 2005). In several reported cases, superinfection has resulted in recombination between the initial and the secondary strains (Fang *et al.*, 2004; McCutchan *et al.*, 2005; Yang *et al.*, 2005).

3. Superinfection: Implications for Vaccine Development

The majority of superinfection appears to has occurred in the early stage of infection. In contrast, several population-based studies reported the rarity of superinfection during chronic infections (Gonzales *et al.*, 2003; Tsui *et al.*, 2004). The reported rarity of superinfection during chronic HIV-1 infection may reflect the time required for the immune responses to mature and may suggest that immune responses in the infected host could protect against superinfection. This may offer hope for an effective vaccine against HIV-1. However, results from several published studies appear to indicate that even strong $CD8^+$ T-cell-mediated responses against the initial infection may not be sufficient for the protection against superinfection (Altfeld *et al.*, 2002; Jost *et al.*, 2002; Yang *et al.*, 2005). Moreover, in most reported cases of superinfection, patients have experienced a decrease in $CD4^+$ cell count and increase in HIV load, accelerating disease progression (Gottlieb *et al.*, 2004; Jost *et al.*, 2002; Smith *et al.*, 2005). The knowledge of superinfection is thus vital to understand the changes in viral pathogenesis and the host immunity and provides the important insights into future vaccine strategies.

C. Biological Implications of Recombination

Recombinant viruses may have certain advantages over the parental strain, including modifications in tropism and replication efficiency ("viral fitness"). Under selection pressure imposed by antiretroviral drugs, recombination between strains with different drug sensitivity resulted in new HIV-1 variants with dual or multiple drug resistance (Moutouh *et al.*, 1996). The

discovery of large numbers of recombinant strains clearly suggests that coinfection with different HIV-1 strains is not rare as once thought. The dual infections with different subtypes have been reported in the regions where multiple variants are cocirculating. Furthermore, as described in the previous section, a study showed that HIV-1 superinfection can occur in the setting of a strong and broadly directed virus-specific $CD8^+$ T-cell response (Altfeld *et al.*, 2002; Jost *et al.*, 2002), suggesting that the host immunological responses are not efficient against divergent strains. These findings would provide important implications for vaccine development as well as for the prevention efforts from public health viewpoints.

VIII. Conclusions

Global dissemination of HIVs represents a dramatic and deadly example of recent genome emergence and expansion. As reviewed in this chapter, recent studies revealed that a pandemic HIV strain, HIV-1 group M, began its expansion in human population roughly 70 years ago (early twentieth century), it has been diversifying rapidly, now comprising a number of different subtypes and CRFs, and that new recombinant strains are arising continually, becoming a powerful force in global HIV-1 spread. Studies also provide information to delineate the mechanism of viral evolution and for the studies on biological features of HIV strains related to pathogenecity and disease progression. However, the biological significance of the global diversity of HIV-1 strains remains to be defined. Although the immune correlates for protection are still incompletely understood, the extensive variation of HIVs could probably be important in the formulation of the vaccine immunogens. In conclusion, molecular epidemiological information on the HIV strains is critically important to elucidate the dynamics of HIV spread and to formulate future vaccine and other prevention strategies.

Acknowledgments

We thank Tee Kok Keng, Shigeru Kusagawa, and Midori Kawasaki for assistance with the manuscript and references, and Drs. Kuan-Teh Jeang and Naoki Yamamoto for their encouragement and advice. We acknowledge grant support from Ministry of Health, Labour and Welfare, Ministry of Education, Science and Technology in Japan and Japanese Foundation for AIDS Prevention. We also thank our collaborators and colleagues in Asia.

References

Alaeus, A., Lidman, K., Bjorkman, A., Giesecke, J., and Albert, J. (1999). Similar rate of disease progression among individuals infected with HIV-1 genetic subtypes A-D. *AIDS* **13**, 901–907.

Altfeld, M., Allen, T. M., Yu, X. G., Johnston, M. N., Agrawal, D., Korber, B. T., Montefiori, D. C., O'Connor, D. H., Davis, B. T., Lee, P. K., Maier, E. L., Harlow, J., *et al.* (2002). HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature* **420**, 434–439.

Amornkul, P. N., Tansuphasawadikul, S., Limpakarnjanarat, K., Likanonsakul, S., Young, N., Eampokalap, B., Kaewkungwal, J., Naiwatanakul, T., Von Bargen, J., Hu, D. J., and Mastro, T. D. (1999). Clinical disease associated with HIV-1 subtype B′ and E infection among 2104 patients in Thailand. *AIDS* **13**, 1963–1969.

Berry, N., Ariyoshi, K., Balfe, P., Tedder, R., and Whittle, H. (2001). Sequence specificity of the human immunodeficiency virus type 2 (HIV-2) long terminal repeat 3 region *in vivo* allows subtyping of the principal HIV-2 viral subtypes A and B. *AIDS Res. Hum. Retroviruses* **17**, 263–267.

Blackard, J. T., Renjifo, B., Fawzi, W., Hertzmark, E., Msamanga, G., Mwakagile, D., Hunter, D., Spiegelman, D., Sharghi, N., Kagoma, C., and Essex, M. (2001). HIV-1 LTR subtype and perinatal transmission. *Virology* **287**, 261–265.

Carr, J. K., Salminen, M. O., Albert, J., Sanders-Buell, E., Gotte, D., Birx, D. L., and McCutchan, F. E. (1998). Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. *Virology* **247**, 22–31.

Casado, G., Thomson, M. M., Sierra, M., and Najera, R. (2005). Identification of a novel HIV-1 circulating ADG intersubtype recombinant form (CRF19_cpx) in Cuba. *J. Acquir. Immune Defic. Syndr.* **40**, 532–537.

Charneau, P., Borman, A. M., Quillent, C., Guetard, D., Chamaret, S., Cohen, J., Remy, G., Montagnier, L., and Clavel, F. (1994). Isolation and envelope sequence of a highly divergent HIV-1 isolate: Definition of a new HIV-1 group. *Virology* **205**, 247–253.

Cilla, G., Rodes, B., Perez-Trallero, E., Arrizabalaga, J., and Soriano, V. (2001). Molecular evidence of homosexual transmission of HIV type 2 in Spain. *AIDS Res. Hum. Retroviruses* **17**, 417–422.

Corbet, S., Muller-Trutwin, M. C., Versmisse, P., Delarue, S., Ayouba, A., Lewis, J., Brunak, S., Martin, P., Brun-Vezinet, F., Simon, F., Barre-Sinoussi, F., and Mauclere, P. (2000). *env* sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. *J. Virol.* **74**, 529–534.

Cuevas, M. T., Ruibal, I., Villahermosa, M. L., Diaz, H., Delgado, E., Parga, E. V., Perez-Alvarez, L., de Armas, M. B., Cuevas, L., Medrano, L., Noa, E., Osmanov, S., *et al.* (2002). High HIV-1 genetic diversity in Cuba. *AIDS* **16**, 1643–1653.

Damond, F., Worobey, M., Campa, P., Farfara, I., Colin, G., Matheron, S., Brun-Vezinet, F., Robertson, D. L., and Simon, F. (2004). Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification. *AIDS Res. Hum. Retroviruses* **20**, 666–672.

De Leys, R., Vanderborght, B., Vanden Haesevelde, M., Heyndrickx, L., van Geel, A., Wauters, C., Bernaerts, R., Saman, E., Nijs, P., Willems, B., Taelman, H., van der Groen, G., *et al.* (1990). Isolation and partial characterization of an unusual human immunodeficiency retrovirus from two persons of west-central African origin. *J. Virol.* **64**, 1207–1216.

Delgado, E., Thomson, M. M., Villahermosa, M. L., Sierra, M., Ocampo, A., Miralles, C., Rodriguez-Perez, R., Diz-Aren, J., Ojea-de Castro, R., Losada, E., Cuevas, M. T., Vázquez-de, E., *et al.* (2002). Identification of a newly characterized HIV-1 BG intersubtype circulating recombinant form in Galicia, Spain, which exhibits a pseudotype-like virion structure. *J. Acquir. Immune Defic. Syndr.* **29**, 536–543.

Delwart, E. L., Shpaer, E. G., Louwagie, J., McCutchan, F. E., Grez, M., Rubsamen-Waigmann, H., and Mullins, J. I. (1993). Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 env genes. *Science* **262**, 1257–1261.

Diaz, R. S., Sabino, E. C., Mayer, A., Mosley, J. W., and Busch, M. P. (1995). Dual human immunodeficiency virus type 1 infection and recombination in a dually exposed transfusion recipient. The Transfusion Safety Study Group. *J. Virol.* **69**, 3273–3281.

Esparza, J., and Bhamarapravati, N. (2000). Accelerating the development and future availability of HIV-1 vaccines: Why, when, where, and how? *Lancet* **355**, 2061–2066.

Esteves, A., Parreira, R., Venenno, T., Franco, M., Piedade, J., Germano De Sousa, J., and Canas-Ferreira, W. F. (2002). Molecular epidemiology of HIV type 1 infection in Portugal: High prevalence of non-B subtypes. *AIDS Res. Hum. Retroviruses* **18**, 313–325.

Fang, G., Weiser, B., Kuiken, C., Philpott, S. M., Rowland-Jones, S., Plummer, F., Kimani, J., Shi, B., Kaul, R., Bwayo, J., Anzala, O., and Burger, H. (2004). Recombination following superinfection by HIV-1. *AIDS* **18**, 153–159.

Galai, N., Kalinkovich, A., Burstein, R., Vlahov, D., and Bentwich, Z. (1997). African HIV-1 subtype C and rate of progression among Ethiopian immigrants in Israel. *Lancet* **349**, 180–181.

Gao, F., Yue, L., White, A. T., Pappas, P. G., Barchue, J., Hanson, A. P., Greene, B. M., Sharp, P. M., Shaw, G. M., and Hahn, B. H. (1992). Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. *Nature* **358**, 495–499.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436–441.

Gonzales, M. J., Delwart, E., Rhee, S. Y., Tsui, R., Zolopa, A. R., Taylor, J., and Shafer, R. W. (2003). Lack of detectable human immunodeficiency virus type 1 superinfection during 1072 person-years of observation. *J. Infect. Dis.* **188**, 397–405.

Gottlieb, G. S., Nickle, D. C., Jensen, M. A., Wong, K. G., Grobler, J., Li, F., Liu, S. L., Rademeyer, C., Learn, G. H., Karim, S. S., Williamson, C., Corey, L., *et al.* (2004). Dual HIV-1 infection associated with rapid disease progression. *Lancet* **363**, 619–622.

Grez, M., Dietrich, U., Balfe, P., von Briesen, H., Maniar, J. K., Mahambre, G., Delwart, E. L., Mullins, J. I., and Rubsamen-Waigmann, H. (1994). Genetic analysis of human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) mixed infections in India reveals a recent spread of HIV-1 and HIV-2 from a single ancestor for each of these viruses. *J. Virol.* **68**, 2161–2168.

Gurtler, L. (1996). Difficulties and strategies of HIV diagnosis. *Lancet* **348**, 176–179.

Gurtler, L. G., Hauser, P. H., Eberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J. M., and Kaptue, L. (1994). A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J. Virol.* **68**, 1581–1585.

Hahn, B. H., Shaw, G. M., De Cock, K. M., and Sharp, P. M. (2000). AIDS as a zoonosis: Scientific and public health implications. *Science* **287**, 607–614.

Hoelscher, M., Kim, B., Maboko, L., Mhalu, F., von Sonnenburg, F., Birx, D. L., and McCutchan, F. E. (2001). High proportion of unrelated HIV-1 intersubtype recombinants in the Mbeya region of southwest Tanzania. *AIDS* **15**, 1461–1470.

Janini, L. M., Tanuri, A., Schechter, M., Peralta, J. M., Vicente, A. C., Dela Torre, N., Pieniazek, N. J., Luo, C. C., Ramos, A., Soriano, V., Schochetman, G., Rayfield, M. A., *et al.* (1998). Horizontal and vertical transmission of human immunodeficiency virus type 1 dual infections caused by viruses of subtypes B and C. *J. Infect. Dis.* **177**, 227–231.

Jost, S., Bernard, M. C., Kaiser, L., Yerly, S., Hirschel, B., Samri, A., Autran, B., Goh, L. E., and Perrin, L. (2002). A patient with HIV-1 superinfection. *N. Engl. J. Med.* **347**, 731–736.

Kaleebu, P., French, N., Mahe, C., Yirrell, D., Watera, C., Lyagoba, F., Nakiyingi, J., Rutebemberwa, A., Morgan, D., Weber, J., Gilks, C., and Whitworth, J. (2002). Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. *J. Infect. Dis.* **185**, 1244–1250.

Kalish, M. L., Baldwin, A., Raktham, S., Wasi, C., Luo, C. C., Schochetman, G., Mastro, T. D., Young, N., Vanichseni, S., Rubsamen-Waigmann, H., von Briesen, H., Mullins, E., *et al.*

(1995). The evolving molecular epidemiology of HIV-1 envelope subtypes in injecting drug users in Bangkok, Thailand: Implications for HIV vaccine trials. *AIDS* **9**, 851–857.

Kalish, M. L., Robbins, K. E., Pieniazek, D., Schaefer, A., Nzilambi, N., Quinn, T. C., St. Louis, M. E., Youngpairoj, A. S., Phillips, J., Jaffe, H. W., and Folks, T. M. (2004). Recombinant viruses and early global HIV-1 epidemic. *Emerg. Infect. Dis.* **10**, 1227–1234.

Kanki, P. J., Travers, K. U., Mboup, S., Hsieh, C. C., Marlink, R. G., Gueye-Ndiaye, A., Siby, T., Thior, I., Hernandez-Avila, M., Sankale, J. L., Ndoye, I., and Essex, M. E. (1994). Slower heterosexual spread of HIV-2 than HIV-1. *Lancet* **343**, 943–946.

Kanki, P. J., Hamel, D. J., Sankale, J. L., Hsieh, C., Thior, I., Barin, F., Woodcock, S. A., Gueye-Ndiaye, A., Zhang, E., Montano, M., Siby, T., Marlink, R., *et al.* (1999). Human immunodeficiency virus type 1 subtypes differ in disease progression. *J. Infect. Dis.* **179**, 68–73.

Kilmarx, P. H., Limpakarnjanarat, K., Kaewkungwal, J., Srismith, R., Saisorn, S., Uthaivoravit, W., Young, N. L., and Mastro, T. D. (2000). Disease progression and survival with human immunodeficiency virus type 1 subtype E infection among female sex workers in Thailand. *J. Infect. Dis.* **181**, 1598–1606.

Kim, S. S., Kim, E. Y., Park, K. Y., Suh, S. D., Park, H. K., Shin, Y. O., Bae, M., and Lee, J. S. (2000). Introduction of human immunodeficiency virus 2 infection into South Korea. *Acta Virol.* **44**, 15–22.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S., and Bhattacharya, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**, 1789–1796.

Kusagawa, S., Sato, H., Watanabe, S., Nohtomi, K., Kato, K., Shino, T., Thwe, M., Oo, K. Y., Lwin, S., Mra, R., Kywe, B., Yamazaki, S., *et al.* (1998). Genetic and serologic characterization of HIV type 1 prevailing in Myanmar (Burma). *AIDS Res. Hum. Retroviruses* **14**, 1379–1385.

Kusagawa, S., Imamura, Y., Yasuoka, A., Hoshino, H., Oka, S., and Takebe, Y. (2003). Identification of HIN type 2 subtype B transmission in East Africa. *AIDS Res. Hum. Retroviruses* **19**, 1045–1049.

Lemey, P., Pybus, O. G., Wang, B., Saksena, N. K., Salemi, M., and Vandamme, A. M. (2003). Tracing the origin and history of the HIV-2 epidemic. *Proc. Natl. Acad. Sci. USA* **100**, 6588–6592.

Liitsola, K., Tashkinova, I., Laukkanen, T., Korovina, G., Smolskaja, T., Momot, O., Mashkilleyson, N., Chaplinskas, S., Brummer-Korvenkontio, H., Vanhatalo, J., Leinikki, P., and Salminen, M. O. (1998). HIV-1 genetic subtype A/B recombinant strain causing an explosive epidemic in injecting drug users in Kaliningrad. *AIDS* **12**, 1907–1919.

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W., and Ray, S. C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **73**, 152–160.

Loussert-Ajaka, I., Chaix, M. L., Korber, B., Letourneur, F., Gomas, E., Allen, E., Ly, T. D., Brun-Vezinet, F., Simon, F., and Saragosti, S. (1995). Variability of human immunodeficiency virus type 1 group O strains isolated from Cameroonian patients living in France. *J. Virol.* **69**, 5640–5649.

Malim, M. H., and Emerman, M. (2001). HIV-1 sequence variation: Drift, shift, and attenuation. *Cell* **104**, 469–472.

McCutchan, F. E. (2000). Understanding the genetic diversity of HIV-1. *AIDS* **14**(Suppl 3), S31–S44.

McCutchan, F. E., Carr, J. K., Bajani, M., Sanders-Buell, E., Harry, T. O., Stoeckli, T. C., Robbins, K. E., Gashau, W., Nasidi, A., Janssens, W., and Kalish, M. L. (1999). Subtype G and multiple forms of A/G intersubtype recombinant human immunodeficiency virus type 1 in Nigeria. *Virology* **254**, 226–234.

McCutchan, F. E., Hoelscher, M., Tovanabutra, S., Piyasirisilp, S., Sanders-Buell, E., Ramos, G., Jagodzinski, L., Polonis, V., Maboko, L., Mmbando, D., Hoffmann, O., Riedner, G., *et al.* (2005). In-depth analysis of a heterosexually acquired human immunodeficiency virus type 1 superinfection: Evolution, temporal fluctuation, and intercompartment dynamics from the seronegative window period through 30 months postinfection. *J. Virol.* **79**, 11693–11704.

Mellquist, J. L., Becker-Pergola, G., Gu, J., Guay, L., Himes, L., Kataaha, P., Mmiro, F., Ndugwa, C., Jackson, J. B., and Eshleman, S. H. (1999). Dual transmission of subtype A and D HIV type 1 viruses from a Ugandan woman to her infant. *AIDS Res. Hum. Retroviruses* **15**, 217–221.

Montano, M. A., Nixon, C. P., Ndung'u, T., Bussmann, H., Novitsky, V. A., Dickman, D., and Essex, M. (2000). Elevated tumor necrosis factor-alpha activation of human immunodeficiency virus type 1 subtype C in Southern Africa is associated with an NF-kappaB enhancer gain-of-function. *J. Infect. Dis.* **181**, 76–81.

Motomura, K., Kusagawa, S., Kato, K., Nohtomi, K., Lwin, H. H., Tun, K. M., Thwe, M., Oo, K. Y., Lwin, S., Kyaw, O., Zaw, M., Nagai, Y., *et al.* (2000). Emergence of new forms of human immunodeficiency virus type 1 intersubtype recombinants in central Myanmar. *AIDS Res. Hum. Retroviruses* **16**, 1831–1843.

Motomura, K., Kusagawa, S., Lwin, H. H., Thwe, M., Kato, K., Oishi, K., Yamamoto, N., Zaw, M., Nagatake, T., and Takebe, Y. (2003). Different subtype distributions in two cities in Myanmar: Evidence for independent clusters of HIV-1 transmission. *AIDS* **17**, 633–636.

Moutouh, L., Corbeil, J., and Richman, D. D. (1996). Recombination leads to the rapid emergence of HIV-1 dually resistant mutants under selective drug pressure. *Proc. Natl. Acad. Sci. USA* **93**, 6106–6111.

Nam, J. G., Kim, G. J., Baek, J. Y., Suh, S. D., Kee, M. K., Lee, J. S., and Kim, S. S. (2006). Molecular investigation of human immunodeficiency virus type 2 subtype a cases in South Korea. *J. Clin. Microbiol.* **44**, 1543–1546.

Neilson, J. R., John, G. C., Carr, J. K., Lewis, P., Kreiss, J. K., Jackson, S., Nduati, R. W., Mbori-Ngacha, D., Panteleeff, D. D., Bodrug, S., Giachetti, C., Bott, M. A., *et al.* (1999). Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. *J. Virol.* **73**, 4393–4403.

Novitsky, V. A., Montano, M. A., McLane, M. F., Renjifo, B., Vannberg, F., Foley, B. T., Ndung'u, T. P., Rahman, M., Makhema, M. J., Marlink, R., and Essex, M. (1999). Molecular cloning and phylogenetic analysis of human immunodeficiency virus type 1 subtype C: A set of 23 full-length clones from Botswana. *J. Virol.* **73**, 4427–4432.

Ou, C. Y., Takebe, Y., Luo, C. C., Kalish, M., Auwanit, W., Bandea, C., DeLaTorre, N., Moore, J. L., Schochetman, G., Yamazaki, S., Gayle, H. D., Young, N. L., *et al.* (1992). Wide distribution of two subtypes of HIV-1 in Thailand. *AIDS Res. Hum. Retroviruses* **8**, 1471–1472.

Ou, C. Y., Takebe, Y., Weniger, B. G., Luo, C. C., Kalish, M. L., Auwanit, W., Yamazaki, S., Gayle, H. D., Young, N. L., and Schochetman, G. (1993). Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. *Lancet* **341**, 1171–1174.

Pau, C. P., Lee-Thomas, S., Auwanit, W., George, J. R., Ou, C. Y., Parekh, B. S., Granade, T. C., Holloman, D. L., Phillips, S., Schochetman, G., Young, N. L., Takebe, Y., *et al.* (1993). Highly specific V3 peptide enzyme immunoassay for serotyping HIV-1 specimens from Thailand. *AIDS* **7**, 337–340.

Pau, C. P., Kai, M., Holloman-Candal, D. L., Luo, C. C., Kalish, M. L., Schochetman, G., Byers, B., and George, J. R. (1994). Antigenic variation and serotyping of HIV type 1 from four World Health Organization-sponsored HIV vaccine sites. WHO network for HIV isolation and characterization. *AIDS Res. Hum. Retroviruses* **10**, 1369–1377.

Peeters, M. (2000). Recombinant HIV sequences: Their role in the global epidemic. *In* "Human Retroviruses and AIDS: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences" (C. Kuiken, B. Foley, B. Hahn, B. Korber, F. McCutchan, P. Marx, J. Mellors, J. Mullins, J. Sidroski, and J. Wolinsky, Eds.), pp. 54–72. Los Alamos National Laboratory, Los Alamos, New Mexico.

Peeters, M., Gueye, A., Mboup, S., Bibollet-Ruche, F., Ekaza, E., Mulanga, C., Ouedrago, R., Gandji, R., Mpele, P., Dibanga, G., Koumare, B., Saidou, M., *et al.* (1997). Geographical distribution of HIV-1 group O viruses in Africa. *AIDS* **11**, 493–498.

Pfutzner, A., Dietrich, U., von Eichel, U., von Briesen, H., Brede, H. D., Maniar, J. K., and Rubsamen-Waigmann, H. (1992). HIV-1 and HIV-2 infections in a high-risk population in Bombay, India: Evidence for the spread of HIV-2 and presence of a divergent HIV-1 subtype. *J. Acquir. Immune Defic. Syndr.* **5**, 972–977.

Piyasirisilp, S., McCutchan, F. E., Carr, J. K., Sanders-Buell, E., Liu, W., Chen, J., Wagner, R., Wolf, H., Shao, Y., Lai, S., Beyrer, C., and Yu, X. F. (2000). A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant. *J. Virol.* **74**, 11286–11295.

Ramos, A., Hu, D. J., Nguyen, L., Phan, K. O., Vanichseni, S., Promadej, N., Choopanya, K., Callahan, M., Young, N. L., McNicholl, J., Mastro, T. D., Folks, T. M., *et al.* (2002). Intersubtype human immunodeficiency virus type 1 superinfection following seroconversion to primary infection in two injection drug users. *J. Virol.* **76**, 7444–7452.

Ray, S. C. (2002). Simplot for *Windows, version3.2* (Distributed by author via http://www.sraymedsomjhmiedu/RaySoft/SimPlot). Johns Hopkins Medical Institutions, Baltimore, MD, USA.

Renjifo, B., Fawzi, W., Mwakagile, D., Hunter, D., Msamanga, G., Spiegelman, D., Garland, M., Kagoma, C., Kim, A., Chaplin, B., Hertzmark, E., and Essex, M. (2001). Differences in perinatal transmission among human immunodeficiency virus type 1 genotypes. *J. Hum. Virol.* **4**, 16–25.

Robertson, D. L., Anderson, J. P., Bradac, J. A., Carr, J. K., Foley, B., Funkhouser, R. K., Gao, F., Hahn, B. H., Kalish, M. L., Kuiken, C., Learn, G. H., Leitner, T., *et al.* (1999). HIV-1 nomenclature proposal: A reference guide to HIV-1 classification. Human retroviruses and AIDS 1999 Los Alamos National Laboratory, NM, pp. 492–505.

Sala, M., Zambruno, G., Vartanian, J. P., Marconi, A., Bertazzoni, U., and Wain-Hobson, S. (1994). Spatial discontinuities in human immunodeficiency virus type 1 quasispecies derived from epidermal Langerhans cells of a patient with AIDS and evidence for double infection. *J. Virol.* **68**, 5280–5283.

Sala, M., Pelletier, E., and Wain-Hobson, S. (1995). HIV-1 gp120 sequences from a doubly infected drug user. *AIDS Res. Hum. Retroviruses* **11**, 653–655.

Schim van der Loeff, M. F., Aaby, P., Xiang, Z., Ariyoshi, K., Wilkins, A., Dias, F., Whittle, H., Breuer, J., I, N. D., Mboup, S., and Kanki, P. J. (1999). Towards a better understanding of the epidemiology of HIV-2. *AIDS* **13**, S69–S84.

Sharp, P. M., Bailes, E., Robertson, D. L., Gao, F., Hahn, B. H., and Ruprecht, R. M. (1999). Origins and evolution of AIDS viruses. *Biol. Bull.* **196**, 338–342.

Sharp, P. M., Shaw, G. M., and Hahn, B. H. (2005). Simian immunodeficiency virus infection of chimpanzees. *J. Virol.* **79**, 3891–3902.

Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Trutwin, M. C., Saragosti, S., Georges-Courbot, M. C., Barre-Sinoussi, F., and Brun-Vezinet, F. (1998). Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat. Med.* **4**, 1032–1037.

Smith, D. M., Richman, D. D., and Little, S. J. (2005). HIV superinfection. *J. Infect. Dis.* **192**, 438–444.

Su, L., Graf, M., Zhang, Y., von Briesen, H., Xing, H., Kostler, J., Melzl, H., Wolf, H., Shao, Y., and Wagner, R. (2000). Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B′) recombinant strain in China. *J. Virol.* **74**, 11367–11376.

Sullivan, M. T., Guido, E. A., Metler, R. P., Schable, C. A., Williams, A. E., Stramer, S. L., Tshimpaka, T., Kambembo, L., Atibu, L., Mama, N., Ilunga, W., Sema, H., *et al.* (1998). Identification and characterization of an HIV-2 antibody-positive blood donor in the United States. *Transfusion* **38**, 189–193.

Takebe, Y., Motomura, K., Tatsumi, M., Lwin, H. H., Zaw, M., and Kusagawa, S. (2003). High prevalence of diverse forms of HIV-1 intersubtype recombinants in Central Myanmar: Geographical hot spot of extensive recombination. *AIDS* **17**, 2077–2087.

Tee, K. K., Li, X. J., Nohtomi, K., Ng, K. P., Kamarulzaman, A., and Takebe, Y. (2006). Identification of a novel circulating recombinant form (CRF33_01B) disseminating widely among various risk populations in Kuala Lumpur, Malaysia. *J. Acquir. Immune Defic. Syndr.* **43**, 523–529.

Thomson, M. M., Villahermosa, M. L., Vazquez-de-Parga, E., Cuevas, M. T., Delgado, E., Manjon, N., Medrano, L., Perez-Alvarez, L., Contreras, G., Carrillo, M. G., Salomon, H., Najera, R., *et al.* (2000). Widespread circulation of a B/F intersubtype recombinant form among HIV-1-infected individuals in Buenos Aires, Argentina. *AIDS* **14**, 897–899.

Thomson, M. M., Delgado, E., Manjon, N., Ocampo, A., Villahermosa, M. L., Marino, A., Herrero, I., Cuevas, M. T., Vazquez-de Parga, E., Perez-Alvarez, L., Medrano, L., Taboada, J. A., *et al.* (2001). HIV-1 genetic diversity in Galicia Spain: BG intersubtype recombinant viruses circulating among injecting drug users. *AIDS* **15**, 509–516.

Thomson, M. M., Delgado, E., Herrero, I., Villahermosa, M. L., Vazquez-de Parga, E., Cuevas, M. T., Carmona, R., Medrano, L., Perez-Alvarez, L., Cuevas, L., and Najera, R. (2002). Diversity of mosaic structures and common ancestry of human immunodeficiency virus type 1 BF intersubtype recombinant viruses from Argentina revealed by analysis of near full-length genome sequences. *J. Gen. Virol.* **83**, 107–119.

Thomson, M. M., Casado, G., Posada, D., Sierra, M., and Najera, R. (2005). Identification of a novel HIV-1 complex circulating recombinant form (CRF18_cpx) of Central African origin in Cuba. *AIDS* **19**, 1155–1163.

Tovanabutra, S., Watanaveeradej, V., Viputtikul, K., De Souza, M., Razak, M. H., Suriyanon, V., Jittiwutikarn, J., Sriplienchan, S., Nitayaphan, S., Benenson, M. W., Sirisopana, N., Renzullo, P. O., *et al.* (2003). A new circulating recombinant form, CRF15_01B, reinforces the linkage between IDU and heterosexual epidemics in Thailand. *AIDS Res. Hum. Retroviruses* **19**, 561–567.

Tsui, R., Herring, B. L., Barbour, J. D., Grant, R. M., Bacchetti, P., Kral, A., Edlin, B. R., and Delwart, E. L. (2004). Human immunodeficiency virus type 1 superinfection was not detected following 215 years of injection drug user exposure. *J. Virol.* **78**, 94–103.

UNAIDS/WHO (2005). "AIDS Epidemic Update", December 2005. (www.unaids.org).

Van Heuverswyn, F., Li, Y., Neel, C., Bailes, E., Keele, B. F., Liu, W., Loul, S., Butel, C., Liegeois, F., Bienvenue, Y., Ngolle, E. M., Sharp, P. M., *et al.* (2006). Human immunodeficiency viruses: SIV infection in wild gorillas. *Nature* **444**, 164.

Vanden Haesevelde, M., Decourt, J. L., De Leys, R. J., Vanderborght, B., van der Groen, G., van Heuverswijn, H., and Saman, E. (1994). Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. *J. Virol.* **68**, 1586–1596.

Vidal, N., Peeters, M., Mulanga-Kabeya, C., Nzilambi, N., Robertson, D., Ilunga, W., Sema, H., Tshimanga, K., Bongo, B., and Delaporte, E. (2000). Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J. Virol.* **74**, 10498–10507.

Weniger, B. G., and Brown, T. (1996). The march of AIDS through Asia. *N. Engl. J. Med.* **335**, 343–345.

Weniger, B. G., Takebe, Y., Ou, C. Y., and Yamazaki, S. (1994). The molecular epidemiology of HIV in Asia. *AIDS* **8**(Suppl 2), S13–S28.

Yang, R., Xia, X., Kusagawa, S., Zhang, C., Ben, K., and Takebe, Y. (2002). On-going generation of multiple forms of HIV-1 intersubtype recombinants in the Yunnan Province of China. *AIDS* **16**, 1401–1407.

Yang, R., Kusagawa, S., Zhang, C., Xia, X., Ben, K., and Takebe, Y. (2003). Identification and characterization of a new class of human immunodeficiency virus type 1 recombinants comprised of two circulating recombinant forms, CRF07_BC and CRF08_BC, in China. *J. Virol.* **77**, 685–695.

Yang, O. O., Daar, E. S., Jamieson, B. D., Balamurugan, A., Smith, D. M., Pitt, J. A., Petropoulos, C. J., Richman, D. D., Little, S. J., and Brown, A. J. (2005). Human immunodeficiency virus type 1 clade B superinfection: Evidence for differential immune containment of distinct clade B strains. *J. Virol.* **79**, 860–868.

Zhu, T., Wang, N., Carr, A., Wolinsky, S., and Ho, D. D. (1995). Evidence for coinfection by multiple strains of human immunodeficiency virus type 1 subtype B in an acute seroconvertor. *J. Virol.* **69**, 1324–1327.

Zhu, T., Korber, B. T., Nahmias, A. J., Hooper, E., Sharp, P. M., and Ho, D. D. (1998). An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* **391**, 594–597.

This page intentionally left blank

Erin-Margaret Murphy*, Humberto R. Jimenez*,†, and Stephen M. Smith*,‡

*Saint Michael's Medical Center, Newark, New Jersey 07102

†Ernest Mario School of Pharmacy, Piscataway, New Jersey 08854

‡The New Jersey Medical School, Newark, New Jersey 07103

Current Clinical Treatments of AIDS

I. Chapter Overview

The treatment of HIV infection has always posed a unique challenge. The number of medications, their various formulations and combinations, their potential toxicities, and drug–drug interactions make HIV one of the most difficult diseases to manage. In the past 11 years, however, there have been dramatic improvements in HIV therapy. Patients with access to newer medications have drastically reduced rates of HIV-related morbidity and mortality. These patients, largely in the United States and other developed countries, have found their quality of life improved with simpler regimens of less toxic medications. This chapter, in the first section, will focus on each Food and Drug

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56002-3

Administration (FDA)-approved anti-HIV compound in detail. The second section will examine issues of HIV treatment, including initiating therapy, choosing a first regimen, monitoring patients with HIV, avoiding complications of therapy, and when to change. There will also be a description of drugs in the late stages of clinical development. Thanks to the recent advances in HIV therapy, clinicians are no longer plagued with the challenge of juggling toxic medications and failing regimens. To prevent progression to AIDS, it is now most important to convince patients to adhere to these powerful medications.

II. HIV Medications

A. Nucleoside/Nucleotide Reverse Transcriptase Inhibitors

1. Overall

a. Mechanism of Action Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) were the first agents approved by the FDA for the treatment of HIV-1 infections. The life cycle of this double-stranded RNA virus requires it to be transcribed into DNA by the enzyme reverse transcriptase (RT) (Yarchoan *et al.*, 1989). These antiretrovirals are nucleoside analogues that interfere with viral replication at this enzyme. NRTIs passively diffuse into the cell where they are converted into their biologically active forms via triphosphorylation (Gao *et al.*, 1993). The sole nucleotide analogue in this class, tenofovir, is slightly different than other nucleoside analogues. Tenofovir is already monophosphorylated. Therefore, this agent foregoes the first phosphorylation step and needs just the final two to become fully active. In general, nucleoside and nucleotide analogues both exert the same activity once in their active forms. Once activated, NRTIs compete with endogenous nucleosides for incorporation into the DNA chain. In contrast to physiological nucleosides, NRTIs lack a 3′ hydroxyl (–OH) group at the sugar moiety of the deoxynucleotide that is essential for the linking of additional nucleosides. Consequently, the inclusion of these drugs into a growing DNA chain leads to its termination. In the United States, there are seven FDA-approved NRTIs on the market. NRTIs have activity versus HIV-1 and HIV-2 strains.

b. Adverse Effects Besides having a similar mechanism of action, NRTIs also share certain toxicities. Most of these class-associated adverse effects are at least partially attributed to mitochondrial dysfunction caused by NRTIs (Brinkman *et al.*, 1998). These antiretrovirals have varying levels of affinity for DNA polymerase-γ, a principal enzyme involved in the production of mitochondrial DNA (mtDNA). Inhibiting this enzyme interferes with oxidative phosphorylation and the subsequent production of adenosine triphosphate (ATP).

Events that have been linked to mitochondrial toxicity include asymptomatic hyperlactatemia, acute lactic acidosis, hepatic steatosis, peripheral neuropathy, pancreatitis, and lipoatrophy. Asymptomatic hyperlactatemia in patients has not been predictive of the development of life-threatening lactic acidosis, which has been correlated with hepatic steatosis (Lonergan *et al.*, 2000). Didanosine (ddI), stavudine (d4T), and to lesser extent zidovudine, have a stronger affinity for DNA polymerase-γ than lamivudine (3TC), abacavir (ABC), tenofovir, and emtricitabine (FTC) (Walker *et al.*, 2002). Many of the adverse effects linked to mitochondrial toxicity occur after long-term treatment with NRTIs. For example, lipoatrophy, a wasting of subcutaneous fat in the face and extremities, only develops after years of NRTI therapy. This untoward effect has been especially linked to prolonged d4T therapy.

2. *Thymidine Analogues*

a. Zidovudine

Pharmacokinetics Zidovudine (ZDV) has an oral bioavailability of ~64% and may be taken without regard for meals (Blum *et al.*, 1988). This drug has significant distribution into the cerebrospinal fluid, reaching 50–70% of serum concentrations (Crowe, 1996, p. 31). The serum half-life of ZDV is about 1.1 h. The half-life of intracellular, activated ZDV is 3–4 h, allowing for twice daily (BID) dosing. ZDV is primarily metabolized in the liver via glucuronidation to the inactive compound 3′-azido-3′-deoxy-5′-*O*-β-D-glucopyranuronosylthymidine (GZDV). Both ZDV and GZDV are renally eliminated (16% and 73%, respectively). The current adult dose is 300 mg orally every 12 h. ZDV is available as a single agent, in combination with 3TC (Combivir®) or with both 3TC and ABC (Trizivir®). With the exception of ABC, the daily dosage of all NRTIs should be adjusted in patients with renal impairment (Table I). Based on pharmacokinetic observations, a 50% dose reduction or a decrease in the dosing frequency of ZDV is also recommended in patients with liver cirrhosis (Taburet *et al.*, 1990).

Adverse Effects When ZDV was first introduced, standard doses of up to 1500 mg/day were associated with significant adverse effects. Subsequent studies established that much lower doses were as effective, with patient side effects being drastically reduced. Bone marrow suppression is a dose-dependent toxicity that commonly occurs with ZDV therapy (Richman *et al.*, 1987). Anemia, the most reported of these toxicities, presents as early as two to four weeks after initiation of the drug. Granulocytopenia is also seen, but much less frequent with the doses currently used. These hematologic abnormalities are more pronounced when ZDV is taken concomitantly with other myelosuppressive agents, such as ganciclovir, interferon-α, dapsone, or pyrimethamine. Macrocytosis is seen in most patients and is not related to a vitamin deficiency. This laboratory parameter

TABLE I Nucleoside Reverse Transcriptase Inhibitors

Antiretroviral			
Daily Dose	*Dosing in renal insufficiency*		
Abacavir* 300 mg PO BID	No need for dosage adjustment		
Didanosine		**Dose**	
>60 kg—400 mg PO QD	**CrCl (ml/min)**	**>60 kg**	**<60 kg**
<60 kg—250 mg PO qd	30–59	200 mg	125 mg
	10–29	125 mg	100 mg
	<10**	125 mg	75 mg
Emtricitabine	**CrCl**	**Capsule**	**Solution**
250 mg cap PO qd	30–49	200 mg q48h	120 mg q24h
(or 240 mg oral solution)	15–29	200 mg q72h	80 mg q24h
	<15**	200 mg q96h	60 mg q24h
Lamivudine	**CrCl**	**Dose**	
300 mg PO qd or	30–49	150 mg QD	
150 mg PO BID	15–29	150 mg × 1, then 100 mg QD	
	5–14	150 mg × 1, then 50 mg QD	
	<5**	50 mg × 1, then 25 mg QD	
Stavudine		**Dose**	
>60 kg—40 mg PO BID	**CrCl**	**>60 kg**	**<60 kg**
<60 kg—30 mg PO BID	26–50	20 mg q12h	15 mg q12h
	10–25** or HD	20 mg q24h	15 mg q24h
Tenofovir	**CrCl**	**Dose**	
300 mg PO QD	30–49	300 mg q48h	
	10–29	300 mg twice weekly	
	HD	300 mg weekly	
Zidovudine	**CrCl**	**Dose**	
300 mg PO BID	<15**	100 mg Q8H	

Note: Use of fixed-dose combination NRTI (+/−NNRTI) of: Atripla®, Combivir®, Trizivir®, Epzicom®—not recommended in patients with CrCl <50 ml/min; use of Truvada—not recommended in patients with CrCl <30 ml/min.

*Mild liver dysfunction (Child-Pugh Score 5–6: 200 mg PO BID).

**If patient is on continuous ambulatory peritoneal dialysis (CAPD) or hemodialysis (HD): use same dose as CrCl <10 ml/min [HD = give dose after dialysis on dialysis days].

has been used as a surrogate marker of a patient's compliance to with his or her drug regimen. Other common adverse effects are headache, myalgia, nausea, and dyspepsia. Cases of lactic acidosis associated with hepatic steatosis have been reported with ZDV (Retrovir, 2006).

Drug Interactions *In vitro* studies have shown that inosine monophosphate dehydrogenase inhibitors, such as ribavirin and mycophenolic acid, may interfere with the phosphorylation of ZDV (Hoggard *et al.*, 1995). However, recent data has questioned the potential interaction (Rodriguez-Torres *et al.*, 2005). Nonetheless, the combination of ribavirin and ZDV has been shown to increase the risk of anemia and should be used with caution (Alvarez *et al.*, 2006). The anticonvulsant valproic acid may increase ZDV levels and lead to toxicity (Lertora *et al.*, 1994). Coadministration of ZDV and tipranavir (TPV)/ritonavir (RTV) resulted in a 35% decrease in the area under the concentration curve (AUC) of ZDV (Aptivus, 2005). The clinical significance of this interaction has not been established. The combination of ZDV and the other thymidine analogue, d4T, has an antagonistic effect and they should not be given together.

b. Stavudine

Pharmacokinetics d4T is very well absorbed, with an oral bioavailability of 86% (Dudley *et al.*, 1992). This agent may be taken with or without food. The serum half-life of d4T is 1.1 h, with an intracellular half-life of 3–3.5 h. Approximately 40% of the changed drug is excreted through the kidneys, warranting a dose reduction in patients with renal insufficiency (Table I). Hepatic dysfunction does not require a dose adjustment as d4T is not metabolized by the liver. The recommended dose is 40 mg BID for patients >60 kg and 30 mg BID if <60 kg.

Adverse Effects The main toxicity experienced with d4T is peripheral neuropathy, occurring in up to 13% of patients (Browne *et al.*, 1993). Symptoms are primarily sensory, characterized by tingling, numbing, and pain. The risk of developing this adverse effect is higher in patients also on ddI or other drugs known to cause peripheral neuropathy, patients with advanced disease, or patients with preexisting risk factors for neuropathy. Discontinuation of d4T usually reverses neuropathy. In one study, neuropathy resolved or improved in 61% patients after switching from d4T to tenofovir (Miralles Alvarez *et al.*, 2004). Compared to other NRTIs, hyperlactatemia has been more commonly reported with long-term d4T therapy (Saint-Marc and Touraine, 1999). This side effect may or may not present with symptoms, such as nausea or myalgias (Carr *et al.*, 1998). Hepatic steatosis may present in d4T-treated patients and can potentially lead to the life-threatening lactic acidosis. Unfortunately, acute lactic acidosis may not be predicted by the presence of asymptomatic hyperlactatemia. d4T is also linked with an increased risk of lipoatrophy with prolonged treatment (Podzamczer *et al.*, 2006; Tavassoli *et al.*, 2006). This antiretroviral has been the NRTI most associated with the development of lipoatrophy. Pancreatitis has been described, particularly when this NRTI was coadministered with ddI. Lipid abnormalities, mainly triglyceride and cholesterol elevations, have also been

seen with this drug, particularly when combined with a protease inhibitor (PI) (Schewe *et al.*, 2006). Most of these untoward effects are primarily a consequence of d4T's mitochondrial toxicity and occur more frequently when ddI is the other NRTI in the regimen.

Drug Interactions The coadministration of ZDV and d4T is not recommended, as ZDV competitively inhibits the intracellular phosphorylation of d4T, decreasing its antiretroviral activity (Hoggard *et al.*, 1997). Doxorubicin and ribavirin may also inhibit the phosphorylation of d4T and should be used with caution when treating comorbid conditions such as Kaposi's sarcoma or hepatitis C infections (Zerit, 2006). In addition, ribavirin increases the risk of lactic acidosis in patients on d4T.

3. Cytosine Analogues

a. Lamivudine

Pharmacokinetics 3TC has oral bioavailability of 86% and is not affected by food (van Leeuwen *et al.*, 1992). Its serum half-life is 2.5 h, with an intracellular half-life of 11–14 h. Since the major route of elimination is through the kidneys (70% unchanged drug), dosing regimens should be adjusted in renal impairment. The current dosage is 150 mg orally BID, although studies have demonstrated its efficacy as a once daily drug (Sosa *et al.*, 2005). 3TC is available as a single agent or in combination with ZDV (Combivir®) and ZDV plus ABC (Trizivir®).

Adverse Effects Historically, 3TC has been the best tolerated of the NRTIs. Adverse effects reported are usually mild in nature. Although lactic acidosis and other class-associated toxicities have been reported with 3TC, studies have demonstrated similar side effect rates between patients on 3TC plus ZDV and those on ZDV monotherapy (Eron *et al.*, 1995). Because this agent also has activity against the *Hepatitis B virus* (HBV), its abrupt discontinuation may lead to a severe acute exacerbation of hepatitis B in patients with both viruses (Epivir, 2006).

Drug Interactions Both trimethoprim and sulfamethoxazole can elevate 3TC concentrations through the competitive inhibition of its renal excretion. However, this interaction has not been shown to be clinically important, as countless patients have tolerated the use of these medications concomitantly (Moore *et al.*, 1996).

b. Emtricitabine

Pharmacokinetics FTC has excellent oral bioavailability (93%). No food precautions are needed with this drug (Emtriva, 2006). FTC and 3TC are structurally similar. FTC, however, has longer serum and intracellular half-lives (10 h and over 24 h, respectively). This agent is primarily

eliminated by the kidneys (86% unchanged drug), requiring the dosing interval to be extended in renal insufficiency. FTC is dosed at 200 mg orally once daily and is also available in combination with tenofovir disoproxil fumarate (TDF) (Truvada®) and with both tenofovir and efavirenz (Atripla®). Of note, Atripla® is the first one pill once a day antiretroviral regimen.

Adverse Effects FTC is generally well tolerated. This drug does have a higher incidence of adverse effects than 3TC, with patients experiencing higher incidences of headache, nausea, diarrhea, and rash. Hyperpigmentation of the palms and soles may also occur in FTC therapy. Due to FTC's antiviral activity against HBV, exacerbations of acute hepatitis B have been reported upon its discontinuation (Emtriva, 2006).

Drug Interactions No major drug interactions have been reported with FTC.

4. Guanosine Analogue-Abacavir

a. Pharmacokinetics ABC is very well absorbed, with an oral bioavailability of 83% (Chittick *et al.*, 1999). Food does not have a significant effect on the absorption of ABC. The serum half-life of the drug is about 1.5 h, with an intracellular half-life of 3.3 h. Approximately 18–33% of the serum concentration distributes into the CSF (Foster and Faulds, 1998). ABC undergoes extensively metabolized by alcohol dehydrogenase and glucuronyl transferase. Thus, dosage adjustments are needed in patients with even mild liver disease (Child-Pugh score of 5–6), but not for kidney dysfunction. The recommended dose is 300 mg by mouth BID, although this drug is approved for 600 mg once daily in combination with 3TC (Epzicom®) (Sosa *et al.*, 2005). ABC is also a component for the three-NRTI formulation with ZDV and 3TC (Trizivir®).

b. Adverse Effects The most common adverse effects are nausea, abdominal pain, headache, weakness, and insomnia. These side effects are generally mild and self-limiting. Hypersensitivity reactions are serious complications that occur in ABC-treated patients (5–8%). This reaction first presents with nonspecific symptoms, such as fever (70–80%), rash (70%), gastrointestinal (GI) effects (50%; nausea, vomiting, diarrhea, and/or abdominal pain), lethargy or malaise (40–60%), and upper or lower respiratory effects (18–30%; cough, shortness of breath, and/or sore throat). Overall, 98% of patients experience either fever or rash or both (Easterbrook *et al.*, 2003). Symptoms appear as early as within the first few days and rarely after six weeks of initial therapy. Patients will consistently feel worse after each dose of ABC, but will not experience a severe, anaphylactic event while continuing to take the drug. This life-threatening reaction occurs once the patient discontinues ABC and is then rechallenged with the drug at

a later time. Within hours of ABC reintroduction, the IgE-mediated reaction leads to significant hypotension, facial or throat swelling, respiratory distress, and eventually death without appropriate supportive care and discontinuation of the drug. Consequently, patients with an allergic reaction to ABC should never receive the drug again. Caution should be taken with some of the coformulated agents as they may also contain ABC. Patients with certain genetic mutations, such as the haplotypes HLA-B*5701, HLA-DR7, and HLA-DQ3, are more likely to develop a hypersensitivity reaction (Mallal *et al.*, 2002; Reeves *et al.*, 2006). Recent studies have found black patients to have a lower incidence of this reaction than white patients (Brothers *et al.*, 2006).

c. Drug Interactions TPV significantly decreases ABC levels (Cmax and AUC reduced by 50% and 40%, respectively), although the clinical implications have not been made clear (Aptivus, 2005). Ethanol has been shown to increase ABC serum concentrations and may increase the occurrence of some common side effects (McDowell *et al.*, 2000).

5. Adenosine Analogues

a. Didanosine

Pharmacokinetics ddI has an oral bioavailability of up to 40% when taken on an empty stomach (Lambert *et al.*, 1990; VidexEC, 2006). Gastric acid inactivates ddI, and originally required the drug to be formulated into a pH buffering tablet. Currently, ddI is available in an enteric-coated capsule (Videx EC®). Unlike the original buffered forms, this formulation does not affect the absorption of other medications commonly prescribed to HIV-infected patients. ddI has a serum half-life of 1.5 h. This drug has an intracellular half-life between 8 and 24 h, allowing for once or BID dosing (Hoetelmans *et al.*, 1998). ddI is mostly excreted through the kidneys (55% as unchanged drug), requiring a decrease in dose for patients with renal insufficiency (Table I). The usual dose is 400 mg orally once daily for patients over 60 kg and 250 mg once daily for those under 60 kg. This agent must be taken at least 30 min before or 2 h after a meal for optimal absorption.

Adverse Effects ddI's most serious toxicity, pancreatitis, occurs in about 7% of all treated (Dolin *et al.*, 1995). The risk of pancreatitis is increased among patients with advanced immunosuppression or patients on other drugs that may cause pancreatitis (i.e., ethanol, hydroxyurea, and intravenous pentamidine). Patient symptoms usually resolve upon discontinuation of ddI and after providing supportive care, although fatalities have been described. Peripheral neuropathy may also occur, especially in patients with a prior history of neuropathy, risk factors for developing neuropathy (i.e., diabetes), or taking other neurotoxic drugs (i.e., ganciclovir). This toxicity is reversible upon discontinuation of ddI. Lactic acidosis associated

with fulminant hepatic failure has also been reported (Kahn *et al.*, 1992). Both these adverse effects are dose dependent; thus, drugs that increase ddI concentrations in the body should be used with caution or avoided if possible.

Drug Interactions ddI concentrations in the body are affected by several agents. Tenofovir, ganciclovir, and allopurinol all increase ddI levels and the risk for toxicity via inhibition of purine nucleoside phosphorylase (PNP), a ubiquitous enzyme involved in the metabolism of ddI (Ray *et al.*, 2004). Tenofovir- and ganciclovir-monophosphate both inhibit this enzyme directly. Allopurinol indirectly affects ddI catabolism by inhibiting xanthine oxidase and increasing levels of hypoxanthine, a product of ddI degradation by PNP (Boelaert *et al.*, 2002). Theoretically, this elevated hypoxanthine concentration disrupts the equilibrium of PNP, slowing ddI degradation. Although the tenofovir–ddI interaction is easily corrected by lowering the dose of ddI, this is not a preferred combination as studies consistently demonstrate an inadequate immune response in comparison to other NRTI backbones (Negredo *et al.*, 2004). This blunted CD4 cell response occurs in light of viral suppression and may be due to impaired lymphocyte maturation and differentiation in the presence of excess the active form of ddI (Fletcher, 2006). Ribavirin may also enhance ddI levels (Moreno, 2004). By inhibiting inosine-5′-monophosphate, ribavirin increases amount of phosphate that is available for biological activation of ddI (Balzarini *et al.*, 1991). Hydroxyurea coadministration has led to ddI-induced pancreatitis and should be avoided. TPV may interact with enteric-coated ddI and should be given at least 2 h apart (Roszko *et al.*, 2003).

b. Tenofovir Disoproxil Fumarate

Pharmacokinetics Tenofovir is an acyclic nucleoside phosphonate (nucleotide) analogue of adenosine-5′-monophosphate and requires only two phosphorylation steps to become biologically active. Compared to the poorly absorbed parent drug, the prodrug (TDF) has an oral bioavailability of 25% without food and 39% with a meal (Fung *et al.*, 2002). This agent has a long serum half-life (12–14 h) and an even longer intracellular half-life (Cundy *et al.*, 1998). TDF is primarily eliminated by the kidneys (70–80% unchanged drug) through both glomerular filtration and active secretion by the renal proximal tubule cells, and requires a dose modification if used in patients with renal impairment. The recommended dose is 300 mg by mouth once daily and is available as a single agent or in combination with FTC and with both FTC and efavirenz (EFV).

Adverse Effects Mild-to-moderate GI side effects are commonly experienced by patients on TDF. The most serious toxicities seen with TDF are usually kidney related. Although kidney dysfunction is uncommon (0.85%),

renal failure, Fanconi syndrome, proteinuria, increased serum creatinine, acute tubular necrosis, and proximal tubulopathy have all been associated with this drug (Malik *et al.*, 2005). TDF is structurally similar to some known nephrotoxic agents, adefovir and cidofovir (Lee and Martin, 2006). While TDF is substantially less cytotoxic than the latter two drugs, it can also accumulate in the renal proximal tubules, inhibiting the growth of these cells (Schaaf *et al.*, 2003). Most of these renal events occur after prolonged treatment, although TDF can precipitate acute kidney disease. As with 3TC and FTC, an acute flare up of hepatitis B may be seen when a coinfected patient abruptly discontinues TDF.

Drug Interactions TDF is not metabolized by the cytochrome *P450* system, nor does it exert any affects on the metabolism of other drugs. Nevertheless, TDF does interact with several medications commonly taken by HIV-1-infected patients. TDF significantly increases ddI concentrations, placing patients at risk of developing toxicities. Acyclovir and ganciclovir may decrease TDF clearance by competing for active tubular secretion (Viread, 2006). When given in combination with lopinavir (LPV)/RTV, the TDF AUC is increased by 34% and the LPV AUC is decreased by 15% (Kearney *et al.*, 2001). The clinical relevance is still unknown. Atazanavir (ATV) levels are significantly lowered by TDF. Conversely, the AUC of TDF is increased 24% by ATV (Taburet *et al.*, 2004). No adjustments are recommended for TDF when combined with ATV unless the patient is renally insufficient. TPV coadministration may lead to diminished serum concentration for both agents (Roszko *et al.*, 2003). Even though the following interaction is not pharmacokinetic in nature, regimens including both ABC and TDF appear to be at higher risk of virological failure and the development of resistance.

B. Nonnucleoside Reverse Transcriptase Inhibitors

1. Overall

a. Mechanism of Action Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are noncompetitive inhibitors of RT. These agents bind to a section outside the catalytic site, altering the configuration of the enzyme (De Clercq, 1995). This conformational change inactivates RT. NNRTIs do not require phosphorylation to be active. This drug class displays excellent *in vitro* and *in vivo* activity against HIV-1. However, its activity is typically short-lived in monotherapy as the virus rapidly develops resistance. In most cases, just one codon change, such as K103N, in RT will render the whole class ineffective. Fortunately, there is an NNRTI in development, TMC125 (etravirine), which maintains antiretroviral activity against HIV-1 strains resistant to the available NNRTIs (Cooper *et al.*, 2006). The agents in this class do not have any activity against HIV-2. Historically, NNRTI-based

regimens have had a lower pill burden in comparison to PI-based regimens, although the gap is narrowing. According to recent data, transmission of NNRTI-resistant viral strains has increased (Weinstock *et al.*, 2004; Wensing *et al.*, 2005). Consequently, before they start a patient on a drug in this class, most clinicians test a patient's virus for resistance to NNRTIs.

Unlike NRTIs, there is an association between the serum drug concentrations and the antiviral activity of the NNRTI at the site of action. Therapeutic drug monitoring (TDM) entails determining a serum concentration range at which a drug achieves the desired therapeutic response and/or is associated with the least amount of toxicity. Theoretically, good drug candidates for TDM exhibit measurable and reproducible serum concentrations when achieving the clinical outcome. Since concentration-response data for NNRTIs is available from previous trials, TDM has the potential to become a valuable tool for optimizing antiretroviral therapy (Back *et al.*, 2006; Boffito *et al.*, 2005). The most important parameter appears to be the minimum concentration (Cmin). Patients able to consistently maintain the Cmin of the respective drug above its minimum inhibitory concentration (MIC) achieve higher rates of virological suppression and lower incidence of resistance than patients with subtherapeutic levels. The role for TDM of NNRTIs in the management of HIV patients has not been established.

b. Adverse Effects Overall, NNRTIs are relatively well tolerated. Unlike NRTIs and PIs, these drugs have not been associated with any long-term adverse effects such as metabolic and morphological abnormalities. Rash is the most common side effect with each agent varying in regard to the severity and frequency. Severe and life-threatening skin reactions, such as toxic epidermal necrolysis and Stevens–Johnson syndrome, have been reported. Although severe rash can occur with any agent in this class, it appears more common with nevirapine (NVP)-treated patients.

c. Drug Interactions Each agent in this class is a substrate of the cytochrome *P450* system (CYP), particularly the CYP3A4 isoenzyme. In addition, NNRTIs have the ability to inhibit and/or induce the activity of CYP3A4 and the P450 enzymes. These chemical properties result in complex drug–drug interactions that elevate or reduce drug concentrations of the NNRTI and/or the coadministered medication(s). Some concomitant medications that are likely to interact include the lipid-lowering agents such as simvastatin and fluvastatin, the antifungals itraconazole and voriconazole, antimycobacterial agents such as rifampin and rifabutin, and the anticonvulsants carbamazepine, phenobarbital, and phenytoin. The potential outcomes of such interactions will be discussed in the sections below.

Herbal products should be avoided as their effects on traditional medications have not been extensively researched. The pharmacokinetics and interaction potential of one herbal supplement, St. John's Wort, has been

well studied. The active ingredients in this natural remedy, hyperforin and hypericin, are known to induce the activity of CYP3A4 and the transmembrane pump P-glycoprotein (P-gp) (Greeson *et al.*, 2001). P-gp decreases the absorption of many drugs by pumping them back into the intestinal lumen (Huisman *et al.*, 2000). The concurrent use of St. John's Wort with an NNRTI will result in the diminished absorption and increased metabolism of NNRTIs (Markowitz *et al.*, 2003; Zhou *et al.*, 2004).

2. *Nevirapine*

a. Pharmacokinetics NVP has excellent oral bioavailability (>90%) and may be taken with or without food (Cheeseman *et al.*, 1993). The serum half-life is 25–30 h (Anonymous, 2006). This drug distributes well throughout the body, including into breast milk and crosses the placenta (Viramune, 2005). NVP is metabolized in the liver by CYP3A4. NVP is also a major CYP3A4 inducer and increases the metabolism of countless medications. The bioavailability of NVP is decreased twofold after one to two weeks due to autoinduction of its own metabolism. This is the rationale behind the initial recommended dosage of 200 mg once daily for 14 days, followed by 200 mg BID thereafter. Although the long half-life of this agent suggests once daily dosing an option, this regimen was shown to have a higher occurrence of grade 3 or 4 liver enzyme elevations compared to twice daily regimen (van Leth *et al.*, 2004).

b. Adverse Effects The most common toxicity experienced with NVP therapy is rash (grade 1/2: 13%; grade 3/4: 1.5%) (Viramune, 2005). This reaction typically occurs within the first six weeks of initiating therapy (Montaner *et al.*, 1998). The rash is generally erythematous and maculopapular in nature and distributes throughout the trunk and upper extremities. Although this reaction is usually mild and self-limiting, up to 9% of patients develop a severe rash, including reported cases of toxic epidermal necrolysis and Stevens–Johnson syndrome described (D'Aquila *et al.*, 1996). Women may be at higher risk of severe reactions than men (Bersoff-Matcha *et al.*, 2001). If a patient develops a mild rash within the first two weeks of therapy, the clinician should continue the 200 mg daily dosing until the rash resolves. Severe reactions warrant abrupt discontinuation of NVP. Another major adverse effect is hepatotoxicity (4%; range: 2.5–11%) (Montaner *et al.*, 1998). The severity of these events varies from transient increases in liver transaminases to fulminant hepatic failure. Hepatotoxicity occurs more frequently in women with a pretreatment CD4 >250 cells/mm^3 and men with a pretreatment CD4 >400 cells/mm^3 at the initiation of therapy. Since rash accompanies these hepatic events approximately half the time, liver function tests should be obtained when patients experience a rash. Monitoring should be done for the first 18 weeks, with the greatest risk within the first 6 weeks.

c. Drug Interactions NVP increases the metabolism of many compounds readily taken by HIV-infected patients through the overexpression of CYP3A4 and to a lesser extent 2B6. NVP lowers methadone levels, potentially leading to opiate withdrawal symptoms in certain patients (Ferrari *et al.*, 2004). Ethinyl estradiol serum levels are also reduced when taken in combination with NVP, usually calling for alternate means of contraception (Anonymous, 2006). In general, raising the daily dosage of the concomitant agent is often required to compensate for the reduced serum concentrations. Serum levels of NVP may be decreased by other CYP3A4 inducers such as rifampin and phenytoin. Furthermore, NVP concentrations can be elevated when combined with a major CYP3A4 inhibitor, such as ketoconazole. The concomitant use of NVP and most of these drugs should be avoided if possible. Also, patients should be monitored carefully for any toxicities or signs of a reduced response to therapy. One potential drawback of just monitoring the antiretroviral response is the increased potential for drug resistance in patients with subtherapeutic concentrations. TDM is one option to improve care when certain drugs must be used together. Nonetheless, TDM is not always helpful due to factors such as interpatient variability and the lack of prospective studies showing a clinical benefit with the use of TDM.

NVP has been studied in combination with PIs to treat HIV-1, resulting in many different interactions affecting the serum drug levels of these agents. For most PIs, NVP significantly reduces concentrations and necessitates an increase in the daily dose of the PI. One exception is nelfinavir (NFV), which is not significantly affected by concomitant use of NVP (Viramune, 2005). RTV is only minimally affected by NVP. Serum levels of LPV do not appear to be lower by NVP when using the new tablet formulation of LPV/RTV (Kaletra, 2006). The use of RTV to enhance the serum level of a coadministered PI by inhibiting CYP3A4 metabolism may counteract the effects of NVP. At present, there is insufficient evidence that boosting with RTV completely overcomes the induction effects of NVP.

3. Efavirenz

a. Pharmacokinetics The bioavailability of EFV has not been well documented, although animal studies reported the oral bioavailability as 42% (Balani *et al.*, 1999). Food increases EFV levels and may lead to more adverse events (Sustiva, 2006). For this reason, EFV should be taken on an empty stomach. The serum half-life varies from 52 to 76 h after a single dose and from 40 to 55 h after multiple doses. This wide range in EFV exposure has been linked to genetic polymorphisms, as patients with a CYP2B6 G516T polymorphism are more likely to have prolonged EFV serum concentrations (Haas *et al.*, 2005). It is hepatically metabolized by the P450 isoenzymes CYP3A4 and CYP2B6 (Deeks, 1998). This agent is eliminated in the urine and feces. No specific dosing recommendations are given for patients with renal or hepatic dysfunction. Increased levels of EFV have

recently been observed in patients with advanced liver disease (Barreiro *et al.*, 2007). The standard adult dose is 600 mg orally at bedtime on an empty stomach. EFV is available as a single agent and in combination with FTC and tenofovir (Atripla^{TM}).

b. Adverse Effects The most common toxicities of EFV involve the central nervous system (CNS). Complaints include dizziness, headache, insomnia, somnolence, vivid dreams, and a sense of disengagement (Deeks, 1998; Sustiva, 2006). CNS effects arise early in therapy and usually resolve after a few weeks without discontinuation of EFV. Taking this medication at bedtime improves its tolerability. EFV should be discontinued if these effects do not improve with continued treatment or if patients experience severe neurological complications, such as psychosis or depression. Rash can also occur with EFV, although usually mild to moderate in nature. This reaction generally subsides without requiring discontinuation. Corticosteroids and antihistamines may be used to treat the rash until it resolves. Patients developing skin rashes while on NVP or delavirdine (DLV) may not necessarily develop a reaction with EFV, due to their distinct structures. Elevations in total cholesterol (20–40%) may be seen with this drug, though not as significant as is seen with PIs (Tashima *et al.*, 2003). Teratogenicity is a concern with EFV, as there are reports of birth defects after exposure during the first trimester. In addition, studies in primates found a significant risk of congenital abnormalities. Nevertheless, the true risk of fetal harm is unclear. Women should be screened for pregnancy before initiating EFV and remain on reliable contraceptive therapy throughout.

c. Drug Interactions EFV is a very potent inducer of CYP3A4 and slightly induces CYP2B6. In addition, EFV is a moderate inhibitor of CYP3A4, CYP2C9, and CYP2C19. These characteristics equate to complicated scenarios when this agent is combined with numerous medications. The overall effect of EFV on CYP3A4 is overexpression of the enzyme, leading to decreased serum concentrations of many CYP3A4 substrates, such as simvastatin, methadone, and rifabutin (Sustiva, 2006). Warfarin therapy should be monitored cautiously when coadministered with EFV as warfarin is primarily metabolized by CYP3A4, CYP2C9, and CYP2C19. Ethinyl estradiol concentrations are elevated by EFV's inhibitory properties (Catanzaro *et al.*, 2004). Rifampin, phenytoin, and carbamazepine can significantly decrease EFV concentrations and may be themselves decreased during coadministration with EFV. Voriconazole levels are considerably lowered by EFV, and EFV was also increased during this concurrent therapy (Vfend, 2006).

The combination of a PI and EFV does not appear to yield more clinical benefit than combining any of these agents with an NRTI backbone (Falloon *et al.*, 2000; Riddler *et al.*, 2006; Solas *et al.*, 2004). This is likely due to the major interaction that occurs at the enzyme CYP3A4. EFV significantly

decreases the serum concentrations of most unboosted PIs, with the exception of NFV (la Porte *et al.*, 2004). When EFV is given together with LPV/RTV, the dose of the PI should be increased to 600 mg/150 mg to compensate for the interaction (Kaletra, 2006).

4. Delavirdine

a. Pharmacokinetics DLV is well absorbed, with an oral bioavailability of 85% (Rescriptor, 2006). This agent may be taken without regard for meals. DLV requires an acidic environment to be absorbed and should be used with caution when combined with acid suppressive agents. The serum half-life depends on the dosage frequency. Although the half-life when given once daily is about 2.8 h, the clearance of DLV is prolonged to ~5.8 h when dosed three times daily (TID). This phenomenon may be due, at least partly, by the inhibition of the drug's metabolism by its own action. DLV is primarily metabolized by CYP3A4. The recommended dose is 400 mg orally TID.

b. Adverse Effects The principal side effect with DLV therapy is rash, which occurs in up to 38% of patients (Scott and Perry, 2000). This reaction is usually erythematous, pruritic, and maculopapular, and presents after one to three weeks of initiating therapy. Rash does not appear to be dose dependent, and in most cases, not dose limiting (Davey *et al.*, 1996; Freimuth *et al.*, 1996). The rash disappears within a couple of weeks without any change in therapy in all but 3–4% of patients, who develop a severe reaction that requires discontinuation. Nausea, fatigue, and headache may present with DLV. Hematologic abnormalities (neutropenia, thrombocytopenia, and anemia) have been associated with DLV in investigational trials.

c. Drug Interactions DLV is a major inhibitor of several P450 enzymes, including CYP3A4, CYP2C9, CYP2C19, and CYP2D6. Serum levels of drugs metabolized through these enzymes, such as warfarin, paroxetine, quinidine, nifedipine, and metoprolol, will be elevated while on DLV. Erectile dysfunction agents, such as sildenafil, should be used at the lowest possible dose and with extreme caution. DLV can also boost serum levels of PIs. Antacids and H_2 receptor antagonists should be spaced appropriately when combined with DLV as they lower the absorption of the drug. Proton pump inhibitors, such as omeprazole, should be avoided altogether. Potent CYP3A4 inducers decrease DLV serum levels and should also be avoided.

C. Protease Inhibitors

1. Overall

a. Mechanism of Action The HIV protease enzyme is vital for completion of the replication cycle. After translation of the proviral DNA, a large

polyprotein is created that encompasses both structural and functional proteins. Cleavage of this polyprotein is necessary to separate the proteins, completing the maturation of the virus. Protease exerts its activity on the polyprotein after the viral particles bud off the cell. PIs act by preventing the cleavage of the polyprotein at the gag-pol position, rendering the released virions immature and noninfectious (Deeks and Volberding, 1997; McDonald and Kuritzkes, 1997). In contrast to NRTIs, these agents do not require intracellular activation. PIs exert potent antiretroviral activity against HIV-1 and HIV-2. Analogous to the NNRTI class, there is relationship between the serum concentrations of PIs and their virological response. TDM is a potential strategy that may lead to improved clinical success in the future.

b. Adverse Effects GI side effects, such as nausea, vomiting, diarrhea, bloating, and abdominal pain, occur frequently during PI therapy (Anonymous, 2006). The extent and intensity of these effects varies among the different agents. GI effects tend to minimize after a few weeks on therapy. Tolerability issues continue for many patients on PI-based regimens, affecting the person's quality of life and adherence. PIs have been linked with increasing bleeding risks in hemophiliac patients. Hepatotoxicity has been described with all PIs, although the frequency and severity have varied between agents.

Many of the adverse effects experienced with PI therapy involve metabolic and morphological changes, usually occurring late in treatment. Chronic issues include lipodystrophy, dyslipidemia, insulin resistance, and bone disorders. Although one may occur independently, these complications often present together in individual patients (Morse and Kovacs, 2006). The precise mechanisms of these adverse effects are not well described. Lipodystrophy with PIs typically presents as visceral and dorsocervical fat accumulation. This central deposition of adipose tissue affects a patient's quality of life tremendously. In addition, these drugs may also accelerate the development of the lipoatrophy linked to NRTI therapy.

Since the introduction of PIs, there has been an increased incidence of lipid disorders. Elevations in serum triglycerides, low-density lipoprotein (LDL) cholesterol, and/or total cholesterol occur in up to 60% of patient receiving PI-based therapy (Behrens *et al.*, 1999). As current HIV therapy improves patient's survival and quality of life, these lipid abnormalities are concerning as they have been associated with an increased risk of cardiovascular disease.

Hyperglycemia, impaired glucose tolerance, and insulin resistance have also been linked with PI use. It is speculated that PIs may interfere with glucose transporter-4-mediated glucose transport (Murata *et al.*, 2000). This insulin resistance predisposes patients to the development of diabetes mellitus.

Finally, osteopenia and osteoporosis seem to occur at a higher frequency in HIV-infected patients compared to the rest of the population. Although

described in patients not on antiretroviral therapy, PIs may enhance the prevalence of this adverse effect. Osteonecrosis or avascular necrosis has also been reported in this population (Tebas *et al.*, 2000).

c. Drug Interactions PIs are all major substrates of CYP3A4 and CYP3A5. In addition, these agents all inhibit CYP3A4 to some degree. RTV has the most potent inhibitory properties, and saquinavir (SQV) has the weakest effect on CYP3A4. These properties produce significant elevations in serum concentrations of drugs metabolized by CYP3A4, potentially leading to drug-related toxicities. Conversely, other CYP3A4 inhibitors may increase the levels of PIs. In addition to inhibiting CYP3A4, some PIs have the ability to induce this enzyme as well. TPV is the most potent inducer. Amprenavir (APV) and RTV also have some CYP3A4 induction properties. Rifampin and phenytoin, among other P450 inducers, may decrease PI serum levels when given when these antiretrovirals. The extent to which these medications interact depends on many factors, such as the drug dosage utilized, whether a drug is metabolized through alternate pathways, and the therapeutic range of the drug. These inhibitory and induction properties of PIs make the interplay of these drugs with other CYP3A4 substrates extremely difficult.

A clinically beneficial interaction, discovered in the late 1990s, was the ability of RTV to considerably elevate serum concentrations and prolong the half-lives of other PIs, improving their effectiveness. For example, adding 100 mg of RTV to a therapeutic dose of SQV increases the serum concentrations by 20-fold compared to the SQV alone (Kilby *et al.*, 2002; Merry *et al.*, 1997). Moreover, the "boosting" effect of RTV allows for a lower pill burden and a reduced dosing frequency in other PI regimens, potentially improving adherence (Anonymous, 2006). PI boosting has revolutionized the standard of care for the management of HIV/AIDS. NFV is the only PI that has not demonstrated a clinical benefit when RTV is added to the regimen. The optimal RTV boosting dose ranges from 100 to 400 daily (as single or divided dose) and depends on the pharmacokinetic properties of the agent being boosted. For example, TPV requires the addition of 200 mg of RTV BID to achieve the therapeutic serum level. This high RTV dose is necessary to overcome the potent CYP3A4 induction properties of TPV, which increases the drug's own metabolism. In contrast, ATV requires just 100 mg daily to boost serum levels considerably.

2. Ritonavir

a. Pharmacokinetics RTV is generally well absorbed, with an oral bioavailability of ~70%. Food increases the absorption of the capsule formulation but may decrease the absorption of the oral solution (Norvir, 2006). The significance of this interaction has not been determined. Nonetheless, RTV is recommended to be taken with food as it improves

the GI tolerance. Serum half-life of RTV is 3–5 h. This agent is metabolized hepatically by CYP3A4. The therapeutic dose of RTV is 600 mg orally BID. Currently, RTV is rarely used as the sole PI in a combination regimen. The current role of RTV is a booster for better tolerated PIs. RTV is available as a 100-mg capsule and as a liquid formulation (80 mg/ml). The capsule should be refrigerated but may be left at room temperature for up to 30 days. The oral solution should be stored at room temperature.

b. Adverse Effects GI adverse effects, including nausea, vomiting, diarrhea, taste perversion, and abdominal cramping, are fairly common with RTV (Norvir, 2006). These effects present early and usually wane with continued therapy. Paresthesias, particularly circumoral, have been reported. RTV may cause elevations in liver transaminases and occasionally severe hepatotoxicity. Hypertriglyceridemia and hypercholesterolemia occur at a high incidence in patients on prolonged therapy, even at the boosting doses. Most of the adverse effects above are dose dependent, arising less often and milder at the lower doses.

c. Drug Interactions RTV is one of the most potent inhibitors of CYP3A4, and partially induces this enzyme as well. In addition, this agent inhibits CYP2D6, CYP2C9, CYP2C19, CYP1A2, and CYP2E1. Drug transportation via P-gp is also inhibited by RTV. Glucuronyl transferase, a non-P450 liver enzyme, is overexpressed during RTV therapy (Krikorian and Rudorf, 2005). For these reasons, RTV has a higher incidence of drug interactions than other PIs when coadministered with other medications. Consequently, the use of many drugs should be used with caution or avoided altogether in patients on RTV.

RTV formulations contain ethyl alcohol (12% and 43% for the capsule and oral solution, respectively) and are contraindicated in combination with disulfiram. This combination will trigger a disulfiram-alcohol reaction. Patients will commonly develop nausea, vomiting, dyspnea, tachycardia, palpitations, vertigo, and hypotension, which may lead to life-threatening effects.

3. Saquinavir

a. Pharmacokinetics SQV has an oral bioavailability of 4%; however, the absorption is greatly improved when boosted with 100 mg of RTV (Invirase, 2005). This PI is now only available as a hard capsule as the production of the better absorbed soft gel capsule has been discontinued in the United States. The serum half-life is 1–2 h. RTV boosting extends the half-life of SQV considerably, allowing for BID dosing (and possibly once daily). SQV is metabolized in the liver by CYP3A4 and is the weakest inhibitor of this enzyme among the PI class. The recommended dose is 1000 mg (2 × 500-mg capsules) of SQV with 100 mg of RTV, given BID

within 2 h of a meal. No dose adjustment is needed for renal dysfunction, but should be used with caution in patients with hepatic insufficiency.

b. Adverse Effects The most prevalent toxicities with SQV therapy are GI in nature, and include diarrhea, nausea, dyspepsia, and abdominal pain. The incidence of hypertriglyceridemia may be lessened in boosted SQV in comparison to boosted LPV or TPV (Anonymous, 2006; Youle *et al.*, 2003).

c. Drug Interactions The metabolism of SQV may be increased or decreased when combined with CYP3A4 inhibitors or inducers, respectively. SQV is a moderate inhibitor of CYP3A4 itself and should not be administered with drugs metabolized by this pathway, such as triazolam and quinidine. SQV is no longer recommended without RTV, and thus most of the interactions will likely be attributed to the boosting portion of the regimen.

4. Indinavir

a. Pharmacokinetics Indinavir (IDV) is rapidly absorbed while in a fasting state and has an oral bioavailability of ~60–65% (Crixivan, 2006). A high-calorie meal results in a significant reduction in AUC and maximum concentration (Cmax) 77% and 84%, respectively (Yeh *et al.*, 1998). Administration with a light snack does not lower the bioavailability of IDV. The mean serum half-life is 1.8 h but is prolonged when combined with RTV. IDV is metabolized hepatically by CYP3A4 and primarily eliminated in the feces (83%). The usual dose is 800 mg (two 400-mg capsules) by mouth TID on an empty stomach or with a light meal, and 600 mg TID in patients with mild-to-moderate hepatic insufficiency. Previous trials have shown RTV-boosted IDV to be a potent combination. However, few patients were able to tolerate this regimen at the doses studied. Thus, the optimal doses of IDV or RTV have yet to be determined. One potential advantage of RTV boosting is the removal of food restrictions.

b. Adverse Effects The most serious toxicity with IDV is nephrolithiasis, occurring in about 12.4% of adults and 29% of children (Crixivan, 2006). Although less than 20% of IDV is excreted by the kidney, the unchanged form of the drug readily crystallizes in urine. Crystallization occurs more readily in a urinary pH above 5. This acute renal colic presents with severe flank pain, fever, nausea, vomiting, and hematuria (Hammer *et al.*, 1997; Kopp *et al.*, 1997). Supportive care, mainly hydration and analgesia, is the crucial for treatment of this toxicity. Discontinuation of IDV is usually not necessary. An oral fluid intake of at least 1.5 liter/day is recommended to prevent renal stone formation. Hyperbilirubinemia occurs in ~14% of patients. This elevation in serum indirect bilirubin occurs due to the inhibition of UDP-glucuronyltransferase (UDP) 1A1 by IDV (Zucker *et al.*, 2001).

Although it usually presents as asymptomatic hyperbilirubinemia, yellowing of the skin and/or eyes may occur. This effect may become cosmetic disturbance for patients. Nephrolithiasis and hyperbilirubinemia are both dose dependent and occur more frequently when the IDV exposure is increased by RTV boosting. GI side effects are also common with IDV, especially nausea (12%) and abdominal pain (17%). The accumulation of visceral fat (called "Crixivan belly") was first described in IDV-treated patients.

c. Drug Interactions IDV may interact with acid suppressive agents, such as omeprazole, as it requires an acidic environment for absorption (Burger *et al.*, 1998). IDV may increase serum levels of ethinyl estradiol and norethindrone by 26% and 24%, respectively. The clinical relevance of this interaction has not been well defined. Serum concentrations of CYP3A4 substrates will also be elevated by this agent. IDV serum levels may be increased or decreased when combined with CYP3A4 inhibitors or inducers, respectively.

5. Nelfinavir

a. Pharmacokinetics NFV has a good oral bioavailability (78%), and its absorption is increased—two- to threefold when taken with food (Quart *et al.*, 1995). Serum half-life is 3.5–5 h. NFV is metabolized by the liver via CYP3A4 and CYP2C19. The enzyme CYP2C19 converts NFV into a hydroxylated active metabolite (M8), which has comparable antiviral activity to the parent drug (Hirani *et al.*, 2004). Metabolites produced by CYP3A4 have minimal activity, if any. RTV boosting has not shown to be pharmacokinetically or clinically beneficial with NFV. The recommended adult dose is 1250 mg (2 × 625 mg tablets) BID with a meal or light snack. No specific dosing recommendations are given for renal or hepatic dysfunction as parent drug and metabolites are eliminated in the feces. Among the PIs available of the market, NFV has accrued the most pharmacokinetic and safety data in pregnancy (Anonymous, 2006).

b. Adverse Effects Diarrhea is the most common adverse effect (Viracept, 2006). Mild-to-moderate diarrhea is seen in up to 61% of patients, lessening in intensity after a few weeks of therapy (Markowitz *et al.*, 1998). Episodes of diarrhea may continue indefinitely, requiring discontinuation of the drug in some patients. In most situations, the NFV-induced diarrhea can be managed with the use of over the counter medications, such as loperamide. In one study, the addition of calcium carbonate 500 mg BID minimized diarrhea in patients significantly (Jensen-Fangel *et al.*, 2003).

c. Drug Interactions Similar to other PIs, NFV is also a major inhibitor of CYP3A4. Thus, the coadministration of many drugs should be avoided

during NFV-based antiretroviral therapy. Also, NFV may induce the expression the enzymes CYP2C9, CYP2C19, and glucuronyl transferase (Honda *et al.*, 1999; Krikorian, 2005; Liedtke *et al.*, 2004).

6. Lopinavir

a. Pharmacokinetics According to preliminary animal studies, LPV has a marginal oral bioavailability due to its extensive first-pass metabolism (Kaletra, 2006; Sham *et al.*, 1998). RTV coadministration with LPV vastly increases its absorption and minimizes its initial metabolism. For this reason, LPV is only available on the market as part of a coformulation with RTV. Food does not significantly affect the bioavailability of the new LPV/RTV tablets, although a moderate or high-fat meal increases the AUC and Cmin of LPV in the older capsule form and oral solution (80% and 54%, respectively). The serum half-life is 5–6 h. LPV is a substrate and an inhibitor of CYP3A4. LPV/RTV is the only boosted combination available on the market. The FDA-approved dose is LPV/RTV 400 mg/100 mg by mouth BID with or without food (with food if the oral solution). The capsule and liquid formulation requires refrigeration and maintains stability at room temperature for one and two months, respectively. The new formulation (200-mg LPV/50-mg RTV per tablet) does not require refrigeration. Treatment-naive patients have the option of taking the total dose (four tablets) once daily (Johnson *et al.*, 2006).

b. Adverse Effects Nausea, vomiting, and diarrhea occur commonly with LPV/RTV therapy. Rates of diarrhea were higher with the 800 mg/200 mg once daily regimen in comparison to the divided dose. These GI side effects are minimized with the new tablet formulation. Hypertriglyceridemia and hypercholesterolemia develop in a significant portion of patients.

c. Drug Interactions The liquid formulation of LPV/RTV contains 42.4% ethyl alcohol, and coadministration with disulfiram should be avoided. Moreover, metronidazole should be used with caution in patients of LPV/RTV due to the risk of a disulfiram-like reaction. Medications eliminated via the P450 system will accumulate when combined with LPV/RTV and should be avoided whenever possible.

7. Amprenavir/Fosamprenavir

a. Pharmacokinetics APV is well absorbed, although the absolute oral bioavailability in humans has not been established (Agenerase, 2006). The oral solution is 14% less bioavailable than the capsule formulation. APV may be given with or without a meal. APV should not be administered with meals high in fat content as decreased serum levels are seen. This drug has a serum half-life of 7–11 h. The bioavailability is improved and the half-life is prolonged when this drug is boosted with RTV. APV is metabolized

primarily by CYP3A4 and by glucuronidation to a small extent. The metabolites are eliminated in the feces and urine. The liquid formulation contains a considerable amount of propylene glycol and should not be given to children less than 4 years of age.

In October 2003, a prodrug of APV, called fosamprenavir (FPV), became available. FPV allows for a lower pill burden and better GI tolerability (Gathe *et al.*, 2004). The usual adult dose in treatment-naive patients is FPV/RTV 1400 mg/200 mg by mouth once daily or 700 mg/100 mg BID. Unboosted FPV is an option in treatment-naive patients, especially if the RTV cannot be tolerated (Anonymous, 2006). Recent studies have demonstrated comparable serum concentrations with the reduced dose of RTV (100 mg daily) versus the original 200-mg dose (Hicks *et al.*, 2006; Ruane *et al.*, 2004). In treatment-experienced patients, the recommended dose is FPV/RTV 700 mg/100 mg BID. The APV oral solution is contraindicated in children under the age of 4, pregnant women, and patients with severe renal or hepatic dysfunction. This is due to the large amounts of propylene glycol and may be harmful in these populations.

b. Adverse Effects The principal adverse effects with APV are headache, GI effects (nausea and diarrhea), and rash. Approximately 18% of patients of this drug develop a skin rash. This reaction is usually mild to moderate and is self-limiting in nature. Roughly 1% of patients experience life-threatening reactions, including Stevens–Johnson syndrome. This reaction occurs due to the sulfonamide moiety on the compound. Patients with a documented allergy to other sulfonamide-containing drugs, such as sulfamethoxazole and sulfadiazine, may be more likely to develop this reaction. The prodrug, FPV, has a comparable adverse effect profile, except for a lower incidence of diarrhea (Gathe *et al.*, 2004).

c. Drug Interactions APV is a moderate CYP3A4 inhibitor, but may also overexpress this enzyme. This agent should not be combined with other drugs metabolized by CYP3A4. The oral solution of APV contains propylene glycol and should not be administered with disulfiram, metronidazole, ethyl alcohol, or other alcohol-based products.

8. Atazanavir

a. Pharmacokinetics ATV has a good oral bioavailability, which is significantly enhanced with a light meal (Reyataz, 2006). The serum half-life is about 7 h and is prolonged substantially with RTV coadministration. This drug is metabolized hepatically by CYP3A4 and is a strong inhibitor of this enzyme. The recommended dose is ATV/RTV 300 mg/100 mg by mouth daily. A 300-mg capsule of ATV was recently approved, making the regimen two pills (one ATV and one RTV) once daily in combination with an

NRTI backbone. Treatment-naive patients have the option of an unboosted regimen (ATV 400 mg once daily).

b. Adverse Effects Diarrhea, nausea, and GI side effects are seen with ATV therapy, but less than with other PIs in comparative trials. Hyperbilirubinemia occurs in 35–49% patients. ATV inhibits bilirubin conjugation to a greater degree than IDV. This dose-dependent phenomenon is usually asymptomatic but may cause scleral icterus or jaundice. Patients on the boosted ATV are more likely to have higher, more intense elevations in conjugated bilirubin. ATV should be discontinued in patients with a total bilirubin level $>5\times$ the upper limit of normal (ULN) due to the lack of data on the long-term effects. In comparison to the other members of the PI class, ATV appears to have the least effect on serum lipid levels, particularly triglycerides (Squires *et al.*, 2004). RTV boosting somewhat counteracts the lipid-friendly profile of ATV, although not completely (Johnson *et al.*, 2005).

c. Drug Interactions ATV is a majorinhibitor of CYP3A4. Substrates of this enzyme should not be administered with ATV. If these drugs are combined with ATV, patients should be monitored closely. Acid-suppressive agents significantly reduce the absorption of ATV (Reyataz, 2006). This PI should be administered 2 h before or 1 h after antacids, such as calcium carbonate and aluminum hydroxide. Famotidine and other H_2 receptor blockers should be held at least 10 h before or 2 h after the ATV dose. Proton pump inhibitors are contraindicated altogether. The bioavailability of ATV is also diminished by TDF, resulting in a decreased of 40% and 25% in Cmin and AUC, respectively (Kaul *et al.*, 2003). The mechanism of this interaction is unclear. ATV must be given with RTV 100 mg daily when combined with TDF. However, boosted ATV AUC and Cmin were decreased by ~25% and 23% in the presence of tenofovir (Reyataz, 2006).

9. Tipranavir

a. Pharmacokinetics The oral bioavailability in humans has not been determined. In animal studies, the absolute bioavailability was about 30% (Poppe *et al.*, 1997). When taken with a high-fat meal, the AUC and peak concentration were elevation dramatically (100% and 55%, respectively) (Baldwin *et al.*, 1998). Serum half-life is optimal (~6 h) with the coadministration of RTV 200 mg with each dose (Aptivus, 2005). This high RTV dose is required to overcome the induction properties of TPV against CYP3A4 and the transporter P-gp. As a substrate and potent inducer of CYP3A4, TPV is rapidly metabolized before achieving therapeutic levels without the addition of RTV. This drug has negligible renal clearance and is

predominately excreted unchanged in the feces. TPV has not been evaluated in patients with hepatic dysfunction and should be used with caution in this population. Approved for highly treatment-experienced patients, the usual dose of TPV/RTV is 500 mg/200 mg orally BID with a high-fat meal. These 250-mg capsules require refrigeration and are stable at room temperature for 60 days.

b. Adverse Effects The most prevalent side effects are GI, mainly nausea and diarrhea. Skin rashes, including urticaria, maculopapular rash, and possibly photosensitivity, have been associated with TPV (Aptivus, 2005). Severe reactions, such as Stevens–Johnson syndrome, have also been described. The incidence of rash was higher in women also taking ethinyl estradiol in comparison to the initial data (33% vs 14%, respectively). Like APV, this compound contains a sulfonamide entity and should be used with caution in patients with a confirmed sulfonamide allergy. Dyslipidemia appears more prevalent with TPV than most of PIs, especially hypertriglyceridemia.

The most serious toxicities are hepatoxicity and intracranial hemorrhage, both warranting a black box warning. In clinical trials, TPV-treated patients where twice as likely to have elevated liver enzymes compared to other PIs (24.4% vs 12.8%, respectively). Hepatic decompensation was also reported, occurring more frequently in patients with hepatitis B or C coinfection and/or on other hepatotoxic medications. Fourteen patients have experienced an intracranial hemorrhage event while on TPV/RTV, eight of these episodes were fatal. While almost 7000 patients were treated with TPV without any such episode, caution should be taken with patients with predisposing conditions, such as head trauma, recent neurosurgery, coagulopathy, and hypertension. Of note, monitoring for coagulation parameters did not predict these events.

c. Drug Interactions Since TPV must be taken with RTV, patients will experience similar drug interactions to any RTV-containing regimen. By also possessing CYP3A4 and P-gp induction capabilities, TPV-related interactions may be even more complicated those seen with RTV. During clinical trials, TPV/RTV was shown to dramatically decrease the serum levels of SQV, APV, and LPV when combined as a part of salvage therapy (Walmsley *et al.*, 2004). TPV also decreases the levels of some NRTIs. Concomitant use of TPV and TDF resulted in dose-dependent reductions in the Cmax (Roszko *et al.*, 2003). ABC and ZDV serum levels are also diminished by TPV. The administration of enteric coated ddI together with TPV led to a 34% decrease in the Cmin of TPV and has been attributed to the emulsifying drug delivery of TPV (Fletcher, 2006). ddI should be taken at least 2 h apart from this PI. TPV capsules contain 7% alcohol and should be avoided in patients on disulfiram, cefotetan, or metronidazole therapy.

10. Darunavir

a. Pharmacokinetics Darunavir (DRV) has an oral bioavailability of 37% when given alone, and 82% when RTV 100 mg added to each dose (Prezista, 2006). Food increases both the AUC and the Cmax (30% each) compared to administering the drug on an empty stomach. When taken in combination with RTV, the serum half-life is ~15 h. DRV is metabolized extensively by CYP3A4 and also inhibits the activity of this enzyme. This agent is eliminated in both urine (13.9%) and feces (79.5%). DRV has no dosing recommendations in patients with hepatic dysfunction but should be used with caution in this population. This drug was approved for patients with multi-PI-resistant HIV, though it is currently being studied in treatment-naive patients. The usual dose is DRV/RTV 600 mg/100 mg by mouth BID. DRV is available in 300-mg capsules.

b. Adverse Effects As with most PIs, the most common adverse effects are GI-related, mostly diarrhea and nausea. Skin rash occurred in 7% of patients, with 0.3% requiring discontinuation of the drug. Stevens–Johnson syndrome and erythema multiforme have been reported with DRV. This agent also has a sulfonamide group, warranting caution when used in patients with a sulfonamide allergy. Thus far, DRV does not have any black block warnings, though there is limited experience with this agent. All other class-associated side effects have been described with DRV.

c. Drug Interactions The combination of DRV/RTV has displayed drug interactions similar to other boosted-PI regimens. Caution should be taken when combining this agent to other CYP3A4 substrates. Moreover, CYP3A4 inducers may lower DRV concentrations and should be avoided whenever possible.

11. Fusion Inhibitor-Enfuvirtide (T-20)

a. Mechanism of Action Fusion inhibitor-enfuvirtide (T-20) is the only entry inhibitor currently available in the market. The entry of the HIV-1 virus into a CD4 cell is a multistep process (Kilby and Eron, 2003). The first of these steps is the attachment of a viral envelope glycoprotein (gp120) to CD4. This step leads to a conformational change in gp120, allowing another portion of the glycoprotein to bind to a neighboring chemokine receptor. Once gp120 is bound to both CD4 and one of two chemokine receptors (CCR5 or CXCR4), gp120 rearranges once again. This rearrangement exposes another glycoprotein (gp41), which is made up of a pair of helically coiled heptad repeats (HR-1 and HR-2). Fusion occurs after HR-2 folds over onto HR-1, stabilizing gp41. The collapsing of gp41 brings the viral envelope and the cell membrane into proximity of each other. Enfuvirtide inhibits this final arrangement step, preventing viral and cell membrane fusion, and thus preventing the viral infection.

b. Pharmacokinetics T-20 is a synthetic 36-amino acid oligopeptide and must be administered as a subcutaneous injection. The bioavailability through this route is 84.3% (Fuzeon, 2004). The serum half-life of T-20 is 3.8 h. This agent is broken down in the liver via catabolism to its constituent amino acids. T-20 is approved for highly treatment-experienced patients, typically as part of a salvage regimen. The recommended dose is 90 mcg subcutaneously every 12 h. The dose should be injected into the abdomen, anterior thigh, or upper arm. T-20 is available as a powder in single-use vials and is stable at room temperature. Prior to administration, the drug must be reconstituted with 1.1 ml of sterile water. This solution should be refrigerated and used within 24 h of preparation.

c. Adverse Effects Injection site reactions occur in almost all patients (98%) treated with T-20 (Fuzeon, 2004). Patients experience pruritis, pain, erythema, ecchymosis, induration, nodules, and cysts on this agent. In clinical trials, patients in the T-20 arm had a higher incidence of bacterial pneumonia. Hypersensitivity reactions, including rash, fever, chills, nausea, vomiting, hypotension, and/or increased liver enzymes, have been reported.

d. Drug Interactions No significant interactions have been identified with T-20.

III. HIV Treatment

There are three main questions to consider when treating HIV-1 infection:

1. When should the treatment be started?
2. What treatment regimen should be selected?
3. When should a therapeutic regimen be changed?

There is some controversy surrounding each of these questions. The US Department of Health and Human Services compiles the *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents (the Guidelines)* (Anonymous, 2006). Many clinicians look to these recommendations for guidance when answering these questions. However, clinical data comes out at a faster pace than the Guidelines can be updated. Consequently, HIV caregivers must constantly review the latest data to provide optimal care for their patients. Unlike other areas of medicine, most HIV clinicians cannot wait for peer review publication of data before they change their practice. The term "antiretroviral" is, of course, inaccurate, but is widely used to refer to the medicines, which inhibit HIV-1. These agents are not active against many retroviruses and some are not active against HIV-2.

A. Initiation of Therapy

There are different opinions over whether to start treatment early or wait until later. The true goal of HIV therapy is the preservation of the immune system since the immunodeficiency caused by chronic HIV infection is the main clinical problem. Some experts recommend offering therapy regardless of CD4 count due to the notion that even low-level viremia can have detrimental effects. Others feel deferring treatment until the CD4 has dropped below a certain number since initiation of therapy when the CD4 count is between 200 and 350 is not associated with worse clinical outcomes (Anonymous, 2006). The latter approach reduces the overall exposure of a patient to HIV medications and consequently reduces long-term adverse effects from the medicines. Finally, deferred initiation reduces the overall cost. The Guidelines recommend offering therapy if any of the following three conditions exist:

1. The CD4 count is below 350, or;
2. The viral load is $\geq$100,000 copies/ml, or;
3. The patient has a history of an AIDS-defining illness or severe symptoms of HIV infection regardless of CD4 count.

One important factor that many clinicians overlook is age, which is not mentioned as a factor in the Guidelines. The cascade collaboration used observational data to comprise a short-term estimation of AIDS progression based on CD4 count, viral load, and age (Phillips, 2004). For example, a 25-year-old individual with a CD4 count of 300 and a viral load of 3000 copies/ml (c/ml) has a 0.8% risk of an AIDS-defining event in the next 6 months. The Guidelines recommend that treatment be started in this patient. Whereas a 55-year-old person with a CD4 count of 450 and a viral load of 30,000 c/ml has nearly double the risk at 1.5%, yet, by the Guidelines, this older patient would not be offered therapy. Even after controlling for CD4 count and viral load, a person's relative risk of disease progression increases by 1.23 for every 10 years older.

In essence, the decision when to start anti-HIV medications is one that should be made by the patient after much discussion with his or her clinician. Clearly, if the patient has had an AIDS-defining event or has a CD4 count less than 200, the clinician should strongly recommend anti-HIV therapy. Still, the patient must be ready to comply with the regimen, must have access to the medications and necessary lab work, and must be ready to deal with potential side effects. If the patient's CD4 count is above 350 and his viral load is less than 100,000 c/ml, the patient and the clinician should discuss the pros and cons of early treatment. Finally, if the patient is above 50 years of age, early treatment should be considered.

Once implemented, HIV therapy should be continued for life. The available therapies potently suppress virus replication, but we do not yet know how to achieve eradication of the virus. Current antiretroviral therapy

attacks the HIV virus at different stages in the replication process. These agents do not reach the latently infected reservoir. Although HIV preferentially replicates in activated CD4 cells, the reservoir is made when HIV infects a CD4 cell that is activated but in the process of reverting back to the resting state (Chun *et al.*, 1997). Thus, the viral genome becomes stably integrated making up the latent reservoir. When the CD4 cell is in the resting state, it lacks the host transcription factors necessary for HIV gene expression. This leads to a transcriptionally silent yet stable pool of HIV-infected long standing cells. The latently infected CD4 T-cells activate intermittently and release infectious virus. No therapy yet exists to target this population. In patients who have had successful viral suppression for several years, HIV replication quickly resumes when the therapy is discontinued.

B. Initial Regimen

As of October 2006, the Guidelines recommend that the initial regimen consists of two NRTIs combined with either a PI or an NNRTI and these are delineated further into preferred and alternative choices (Anonymous, 2006).

1. NRTI Choice

These recommendations are based on analysis of multiple trials and expert opinions. However, there are still several decisions that need to be made on an individual basis. It is important to know which trials are being referenced. Some recent trials have yet to influence the Guidelines, but have clinical implications. For example, the Guidelines recommend using two NRTIs paired with either an NNRTI or a PI. ZDV and 3TC (coformulated as Combivir®) are still listed as a preferred NRTI combination. However, most clinicians, based in large part on the recent findings of the clinical trial Study GS934, consider Combivir® an alternative regimen (Gallant *et al.*, 2006). This study directly compared tenofovir/FTC to coformulated ZDV/3TC. This open label trial had a primary endpoint of a viral load <400 c/ml at week 96. 509 HIV-infected, treatment-naive patients with a viral load >10,000 c/ml were randomized 1:1 to either the ZDV/3TC/EFV arm or the tenofovir/FTC/EFV arm. At 96 weeks, 75% of patients in the tenofovir/FTC arm had a viral load <400 c/ml and 67% were at <50 c/ml, whereas 62% and 61% of those in the ZDV/3TC arm had a viral load <400 and <50 c/ml, respectively. Also, the mean absolute increase in CD4 cell count from baseline was 270 cells/mm^3 in the tenofovir/FTC arm and 237 cells/mm^3 in the ZDV/3TC arm. The superior virological response in the tenofovir/FTC arm was due to less discontinuation. The most common reason for discontinuation in the ZDV/3TC arm was anemia.

The 2004 CNA30024 study was also designed to study the NRTI backbone, specifically comparing ZDV to ABC (DeJesus *et al.*, 2004).

The study was a randomized and double blinded. Each patient received 3TC and EFV. 649 HIV-infected, treatment-naive patients were enrolled. Both arms performed similarly; at 48 weeks 70% of patients in the ABC/3TC/EFV arm and 69% in the ZDV/3TC/EFV arm achieved a viral load of <50 c/ml. However, there was a significant difference in CD4 response favoring the ABC arm ($p = 0.0039$). Also, as expected, the toxicities were different between the arms showing more hypersensitivity in the ABC arm and increased anemia, nausea, and fatigue in the ZDV arm.

Together, these recent studies support tenofovir and ABC as first-line agents with either of the cytosine analogues, 3TC, or FTC. The cytosine analogues are very similar and appear to be interchangeable. Most clinicians would consider ZDV a second-line NRTI choice. Also implicit in the Guidelines recommendations is that a cytosine analogue be used as one of the two NRTIs (Anonymous, 2006). This decision was probably made for several reasons. First, most recent studies have used a cytosine analogue. Second, cytosine analogues are very well tolerated and dosed once daily. Third, they provide synergy when used with tenofovir, ABC, or ZDV. Fourth, no other two NRTI combinations have been shown to be more effective. Fifth, each of the three coformulations, which have two NRTIs, includes a cytosine analogue. Although fewer data exist for them, other NRTI combinations have been used successfully.

In addition to these completed trials, there are ongoing trials that are also studying the NRTI backbone. The HEAT study (EPZ 104057) is a 96-week comparison of ABC/3TC (coformulated as Epzicom®) to FTC/tenofovir (coformulated as Truvada) both in combination with LPV/RTV (Anonymous, 2007). The ACTG 5202 is an 1800 patient 96-week trial with 4 arms of FTC/tenofovir or ABC/3TC combined with either EFV or ATV/RTV. Both of these studies are currently open for enrollment and when completed will guide clinicians in the NRTI backbone choice. In addition, the ACTG 5202 will offer valuable information about which third agent to use in combination (Anonymous, 2007).

2. *NNRTI Versus PI*

The choice of NNRTI versus PI is still a matter of debate in naive patients. Many clinicians have felt that a boosted PI is more effective than an NNRTI in patients with viral loads greater than 100,000 c/ml. ACTG 5142 addressed these issues. Completed in 2006, this study compared LPV/r (r = RTV) to EFV (Riddler *et al.*, 2006). There were three arms; LPV/r with two NRTIs, EFV with two NRTIs, and LPV/r plus EFV. LPV/r and EFV have long been the most commonly prescribed agents of their classes, but direct comparison had been lacking. 887 HIV-infected, treatment-naive patients with a viral load >2000 c/ml were enrolled. At 96 weeks, 89% of patients in the EFV/NRTI arm had a viral load <50 c/ml, whereas 83% of patients in the LPV/r/EFV arm and 77% of those in the LPV/r/NRTI had a viral load <50 c/ml.

The virological response difference between the EFV/NRTI and the LPV/r/NRTI arms was statistically significant ($p = 0.003$). In addition to improved virological efficacy, EFV containing arms also showed a longer time to virological failure. The percentage of patients in the LPV/r/NRTI arm that failed by week 96 was 33% as compared to only 24% in the EFV/NRTI arm and 27% in the LPV/r/EFV group. Conversely, the immunologic response, defined as increase in CD4 count, was superior in the LPV/r group. At 96 weeks, there was an increase by 285 cells in this arm, compared to 268 cells in the LPV/r/EFV arm and 241 cells in the EFV arm. The immunologic response differences between either of LPV arms and the EFV/NRTI arm were statistically significant. In those patients who had virological failure in the LPV/r/NRTI arm, 15% NRTI mutations (mostly M184V) developed. In the EFV/NRTI arm, 33% of virological failures developed NRTI mutations (typically M184V and K65R). The NNRTI mutation, K103N, was seen in 48% of patients who failed the EFV/NRTI arm and 69% who failed in the LPV/r/EFV arm. While all three arms showed virological suppression, the likelihood of failure secondary to resistance in the LPV/r/EFV arm makes this combination a suboptimal choice for therapy. EFV and LPV remain equally recommended by the Guidelines. The data from this specific trial deals only with LPV/r and EFV and cannot be applied class wide. The trial offers clinicians invaluable data about these reigning first-line treatment options.

3. *Initial PI*

Within the PI class, clinicians often use LPV/r. One of the original trials designed to study LPV/r was completed in 2002 and compared LPV/r to NFV; both combined with 3TC/d4T (Walmsley *et al.*, 2002). 653 patients were enrolled and randomized 1:1 to each arm for 48 weeks. At 48 weeks, the LPV/r arm was superior to the NFV arm in virological suppression showing 75% of patients with a viral load <400 and 67% at <50 c/ml compared to the NFV arm with only 63% of patients at <400 c/ml and 52% of patients <50 c/ml. In addition, there was a superior performance in time to failure in the LPV/r arm with 84% of patients with persistent virological suppression versus 66% in the NFV arm. This early trial led many clinicians to the conclusion that LPV/r containing regimens are superior to those containing NFV for treatment-naive patients.

Although LPV/r is the most commonly prescribed PI, opinion among clinicians has begun to shift to include some of the other agents in this class as first-line PIs. The KLEAN study was a recent head-to-head comparison of boosted PI regimens: FPV/r versus LPV/r combined with fixed-dose ABC/3TC (Eron *et al.*, 2006). Completed in 2006, this trial enrolled 878 treatment-naive patients randomized 1:1 to each arm. At 48 weeks, there was no difference between the arms in virological suppression (either <400 or <50 c/ml), CD4 count increase or adverse events. Another trial that

compared PIs was the BMS 045 study completed in 2006. This was a 96-week comparison of ATV/r and LPV/r combined with tenofovir plus another NRTI in 358 treatment-experienced patients. Although the study examined treatment-experienced population, it does offer the clinician a useful look at the comparison data. At 96 weeks, there was comparable efficacy and safety with mean virological reductions from baseline of −2.29 and −2.08 log copies/ml in the ATV/r and LPV/r arms, respectively. The data from both of these studies offers reassurance that LPV/r is not the only efficacious boosted PI regimen.

4. Initial NNRTI

When deciding on which NNRTI to select, one may examine the 2NN study, which compared not only EFV to NVP but also EFV combined with NVP. This study was completed in 2004 and enrolled 1216 HIV-infected, treatment-naive patients randomized to 1 of 4 arms: NVP 400 mg daily (QD), NVP 200 mg BID, EFV 600 mg QD, or NVP/EFV 400/800 mg QD (van Leth *et al.*, 2003). Each arm included 3TC/d4T. At 48 weeks, the percentage of patients with a viral load <50 c/ml was 88.7% in the NVP QD arm, 81.5% in the NVP BID arm, 86.8% in the EFV arm, and 79.5% in the NVP/EFV arm. It was found that there was no added advantage to combining NVP and EFV. There was a higher rate of study withdrawal secondary to adverse events in the NVP/EFV arm. Although the difference between the arms is not deemed statistically significant, there is a trend toward the EFV arm being more effective. Also, there was a higher incidence of hepatotoxicity in the NVP containing arms, especially the QD arm. While the efficacy of EFV and NVP were not significantly different, Phase IV data have shown that NVP can cause life-threatening hepatotoxicity. Many clinicians lean toward selecting EFV when choosing from the NRTI class. At the very least, it is imperative to take caution and note the CD4 count of patients when selecting an NRTI. Women with a baseline CD4 count >250 cells/mm^3 and men with a count >400 cells/mm^3 are at a significantly increased risk of rash-associated liver toxicity compared with patients who start NVP treatment with lower CD4 counts. EFV, as in the previous section, must be used with caution in women of child-bearing age. EFV is severely teratogenic in macaques, so women of child-bearing age taking it must avoid pregnancy (Cadman, 1998).

5. Three Class Regimens

The Guidelines recommend using an NRTI backbone combined with either an NNRTI or a PI, but are other regimens acceptable? Is there any added efficacy to using an agent from each of the three classes NRTI/NNRTI/PI? There have been three recent trials designed to look at the 3 class option. The ACTG 384 study published in 2003 compared ZDV/3TC or ddI/d4T plus EFV and/or NFV (Robbins *et al.*, 2003). There was no

difference between 2 class and 3 class regimens. This concept was followed up twice in the INITIO and FIRST trials, both completed in 2006. INITIO found that 3 class (ddI or d4T with EFV and NFV) was no different than 2 class and FIRST also found that 3 class (NRTI and NNRTI and PI—mostly EFV and NFV) was no different than 2 class (MacArthur *et al.*, 2006; Yeni *et al.*, 2006). It is clear to clinicians that there is no added benefit regardless of specific agent to choosing agents from each of the three classes NRTI/NNRT/PI versus selecting the accepted two NRTIs with an NNRTI or a PI.

6. Triple NRTI Therapy

The concept of adding a third NRTI either for class combination or a single class regimen has also had some recent exploration. The ACTG 5095 study looked at time to first virological failure in three arms: ZDV/3TC/ABC, ZDV/3TC with EFV, and ZDV/3TC/ABC with EFV (Gulick *et al.*, 2004). Completed in 2003, there were 1197 patients randomized to 1 of these 3 arms. At 32 weeks, virological failure occurred in 21% of the triple NRTI arm and 11% in the pooled EFV arms. Due to the higher rate of failure, the triple NRTI arm was unblinded and stopped early. When the resistance was analyzed in the triple NRTI group, wild type existed in 22%, M184V in 34%, M184V plus other NRTI mutations in 11%, and NRTI mutations without M184V in 2%. Thirty-one percent of failures were unable to be sequenced. Although much smaller, a pilot study was done assessing the combination of three different NRTIs: ABC, 3TC, and tenofovir. The preliminary data revealed that 11 of the 19 patients had virological failure (Farthing *et al.*, 2003). The failure in the triple NRTI regimens is most likely due to the low genetic barrier to resistance. These two separate trials point to the conclusion that there is no added benefit in adding a third NRTI and furthermore that using an NRTI class only is not recommended due to the earlier progression to virological failure.

7. PI Monotherapy

PI monotherapy is utilized at times by clinicians in the face of NRTI resistance and multiple PI mutations, but is it efficacious in the treatment-naive individual? The latest trial looking at this was the MONARK study completed in 2006. Patients enrolled in this trial were HIV infected, treatment-naive with viral load <100,000 c/ml and CD4 >100 (Delfraissy *et al.*, 2006). 136 subjects were randomized 1:1 to either the LPV/RTV arm or the ZDV/3TC with LPV/RTV. At 48 weeks, 75% of patients in the ZDV/3TC/LPV/RTV achieved virological suppression (<50 c/ml) compared to 71% of those in the LPV/RTV arm. However, when analyzed by OT (on treatment as opposed to ITT or intent to treat) 98% of patients in the ZDV/3TC/LPV/RTV arm were at <50 c/ml compared to 84% on monotherapy. Another look at PI monotherapy was done in the ACTG 5201 study. This was a simplification or switch pilot study looking at ATV/RTV monotherapy

in 36 subjects (Swindells *et al.*, 2006). Subjects with virological suppression for at least 48 weeks on their first PI-based regimen containing 2 NRTIs were enrolled. Participants switched from current PI to ATV/RTV at entry and discontinued the NRTIs after six weeks. At 24 weeks, 91% of patients had a viral load <50 c/ml. Based on the results of these trials, PI monotherapy has been deemed able to achieve virological suppression, but most clinicians reserve PI monotherapy for the treatment-experienced patient.

8. PI Plus NNRTI (NRTI Sparing)

Regarding the question of an NRTI sparing regimen, the results of the ACTG 5142 as discussed earlier point to this as a poor choice due to failure from resistance. However, this combination (LPV/r/EFV) remains an option for those who have failed triple NRTI regimens, such as Trizivir®, and have virological resistance to all NRTIs. Dosing of LPV must be increased to overcome the induction of its metabolism by EFV.

C. Long-Term Management

As above, treatment for HIV, once started, should be administered for life. In the recent past, there has been a considerable amount of interest in the efficacy and safety of structured treatment interruptions (STI). Continuous treatment with these potent agents can lead to toxicities and resistance as well as being quite costly. Thus, strategies that involve cycles of treatment withdrawal and reinitiation were heavily studied in the past two years. Some of these studies looked at the intermittent approach, that is prespecified times of treatment withdrawal, and others focused on the CD4 cell count guided approach. The largest and most recognized of these was the SMART trial.

The SMART trial was commenced in 2001 and was designed to compare two antiretroviral treatment strategies, the drug conservation (DC) strategy and the viral suppression (VS) strategy (El-Sadr *et al.*, 2006). The reasoning behind the SMART study is that the principal goal of HIV therapy has been the preservation of the immune system, as determined by CD4 count. In the DC group, therapy was stopped in patients with a CD4 cell count above 350. Therapy was restarted when the CD4 count fell below 250. By design, patients in the DC group could cycle on and off therapy, based on their CD4 counts. The VS group aimed for sustained virological suppression regardless of CD4 cell count. The trial enrolled 5472 HIV-infected subjects with a CD4 cell count of at least 350 who were then randomized 1:1 to each group.

The SMART trial halted enrollment early due to the higher rates of death and HIV disease progression in the DC group. Unexpectedly, patients in this group also had a higher incidence of adverse events such as cardiovascular events, renal disease, liver disease, and other end organ damage

than those in the VS group. Experts were surprised by these results given the notion that antiretroviral agents are associated with metabolic complications such as lipids abnormalities and decreased kidney and liver function. More importantly, patients in the DC group had a significantly higher mortality rate. Subsequently, based on the SMART study design, it can be concluded that episodic use of antiretroviral therapy based on CD4 cell count levels is inferior to continuous antiretroviral therapy for management.

The results of the SMART trial and other STI studies have not made their way into affecting the Guidelines, but they definitely have clinical implications. Most of the data is leaning toward the conclusion that treatment interruption is not a good strategy and should be used only with great caution. The rebound viremia after treatment interruption is associated with serious related adverse events. The data suggests that HAART should be continued once it is started to reduce said adverse events.

Although the SMART and other STI trials did not examine this issue in any way, their results have prompted many to reexplore the recommendations for treatment initiation. In the SMART trial, DC group patients with baseline CD4 cell counts above 650 still had a significantly higher death rate and increased HIV disease progression. This subgroup analysis suggests that earlier treatment initiation may in fact be beneficial, especially with the newer, better tolerated HIV therapies. The debate on when to initiate treatment is ongoing and the STI trials imply that further clinical research on this topic is still needed.

D. Resistance

Through the use of proper pharmacotherapy, progression to AIDS is now preventable. However, with poor adherence progression is almost inevitable. Patient compliance to the individually designed antiretroviral regimen is essential to the success of sustained virological suppression. Noncompliance leads to suboptimal antiretroviral drug levels, which apply selective pressure causing drug-resistant mutants to preferentially replicate. This viral resistance results in the elimination of available antiretroviral agents to use in future regimens. Other reasons for failure include suboptimal pharmacokinetics (i.e., unsuspected drug–drug interactions), high baseline viral load, active substance abuse, and unknown reasons.

So the question remains, when should the regimen be changed and what should it be changed to? Studies have established that continuous administration of ART in the face of new and persistent viremia increases the number of drug resistance-associated mutations. The more mutations that develop, the fewer drugs will remain active against a patient's virus. Some controversy still exists over when to change therapy and what to change to. Virological, immunologic, and/or clinical failures are all reasons to change therapy. Virological failure is defined by the Guidelines as "HIV RNA level

>400 copies/ml after 24 weeks, >50 copies/ml after 48 weeks, or a repeated HIV RNA level >400 copies/ml after prior suppression of viremia to <400 copies/ml."

In 2007, clinicians have many effective second-line options to choose from. Even when faced with multiresistance virus, the clinician can construct several different regimens. Optimally, three active medications should be prescribed. If this is not possible, then two active agents should be given. If only one active drug is used, there is a good chance that resistance will develop and that drug and perhaps the entire drug class will become ineffective against that patient's virus.

Testing for viral resistance to the anti-HIV drugs is recommended before starting a second or later regimen. There are two distinct types of resistance assays, genotyping and phenotyping. In genotyping, a patient's virus is purified from plasma, viral RNA is extracted and then amplified by RT-PCR. The resulting cDNAs are sequenced en masse. The DNA sequence is converted to encoded amino acids, and the amino acid sequences are then analyzed for mutations associated with resistance to particular drugs. For instance, a viral cDNA sequence which results in the codon for valine at position 184 (instead of the naturally occurring methionine) in *reverse transcriptase* implies resistance to FTC and 3TC. The mutations associated with resistance to NNRTIs and NRTIs are very well studied and understood. The mutations associated with PI resistance are less well understood. Since the sequencing is done en masse, minority species (those <20%) are typically not detected. Genotyping is less expensive than phenotyping and often gives the clinician the needed information.

For patients whose virus has complex mutations in protease, phenotyping may be helpful. Phenotyping is also based on RT-PCR of plasma viral RNA. However, after PCR, single-cycle reporter viruses are produced with patient-derived *protease* and *reverse transcriptase* genes. The recombinant virus is then used to infect a cell line in the presence of increasing amounts of each anti-HIV compound. Resistance is determined by the increase of a drug needed to inhibit reporter gene expression by 50%. For instance, if the recombinant virus with patient-derived *protease* requires 10 times more LPV than wild-type virus to reduce the reporter expression by 50%, then that virus is resistant to LPV. Phenotyping is also not sensitive at detecting minority species of resistant virus. Genotyping and phenotyping offer invaluable information about the patient's viral mutations and susceptibility to different drugs.

E. Drugs in Development

Newly developed agents are often designed for the heavily treatment-experienced patients. For example, the two most recently approved PIs, TPV and DRV, were developed for use in experienced patients and have shown good activity in the face of multiple PI resistance-associated mutations

(Aptivus, 2005; Prezista, 2006). Similarly, novel NNRTIs and integrase inhibitors, as of this writing, are in the advanced stages of drug development. As of January 2007, 35 new compounds are being studied in Phase I or later clinical trials (Companies, 2006). Of course, it is common for investigational agents to never make it out of Phase I or early Phase II due to various problems. Of these investigational agents, there are several agents in existing classes as well as agents with new mechanisms of action. It is the agents with new mechanisms of action that are often the most exciting and closely followed by clinicians.

Also, when all currently available agents have been exhausted, new agents can sometimes be accessed via expanded access programs (EAP). Two agents are currently in EAP stages: TMC-125 (etravirine), which is a novel NNRTI, and MK-0518, which is the first in the new class of agents called integrase inhibitors which are discussed later (Anonymous, 2007). New classes of antiretrovirals that target new or other viral proteins are crucial given the efficacy of viral suppression when two steps of viral replication are blocked. Salvage therapy most often needs to include these newer agents. EAP allow patients access to new drugs, which have completed Phase IIb or III trials and are awaiting FDA approval.

The novel class of integrase inhibitors is showing promising activity against HIV (Anonymous, 2007). Integrase is the last of the three HIV enzymes to be targeted by drug therapy. The exact mechanism of action of integrase is not entirely understood, but it is known that integrase has three domains and that it cleaves the proviral DNA to activate the ends, allowing the proviral DNA to be incorporated into the host cell DNA. HIV integration can be thought of as happening in four steps: preintegration complex formation, 3′ end processing, DNA strand transfer, and repair. Integrase catalyzes the second and third steps. It prepares the viral DNA for insertion by removing a dinucleotide from each 3′ end, which exposes new hydroxyl groups. The preintegration complex formation is then bound to host DNA and the DNA strand transfer is completed. Due to the newly exposed hydroxyl groups, host bonds are broken and new covalent bonds are created between viral and host DNA, thus splicing viral DNA into host DNA. All leftover gaps and free DNA strands are then repaired by host enzymes. A purported mechanism of action of integrase inhibitors is potent and selective inhibition of DNA strand transfer (Grobler *et al.*, 2002; Hazuda *et al.*, 2000). There is very little activity against the 3′ processing. With this strand transfer inhibited, the insertion of the proviral DNA into host DNA fails and the preintegration complex is degraded by host enzymes.

The integrase inhibitors in earlier stages of development include GS-9317, which is shortly going to enter Phase III, and GSK-735, which is currently in Phase IIa. As mentioned earlier, MK-518 is currently available through EAP. This was studied in treatment-naive patients who were randomized to one of the MK-518 doses plus tenofovir/3TC or the EFV/tenofovir/3TC arms.

The currently published 24-week data reveals that >85% of patients had a viral load of <50 c/ml. More so, at 8 weeks, MK-518 was statistically superior to EFV; however, by week 24 they were equivalent. It is currently being studied in the experienced patient population but no data past eight weeks has been published as of yet.

Also currently in development are novel compounds in the entry inhibitor class. Entry of HIV into target cells is the first stage of viral replication, and there was a great deal of enthusiasm over the CCR5 inhibitors. However, only two agents remain in clinical development: vicriviroc in Phase II and maraviroc which will shortly enter EAP (Anonymous, 2007). There have been problems that have slowed the development of these agents including inferior antiviral activity and adverse events, such as hepatotoxicity and malignancy. The development of aplaviroc was discontinued secondary to hepatotoxicity. Although in an early study of vicriviroc several patients developed lymphoma, it remains in development as no causal association has been established. Also, ~50% of patients are dual tropic for X4 and R5 virus, rendering the agent ineffective. As with any novel compound, the long-term safety of these agents has yet to be proven.

For patients who are either ineligible for EAPs or are in a holding regimen until another active agent becomes available 3TC or FTC, monotherapy has been shown to offer some efficacy (Campbell *et al.*, 2005; Castagna *et al.*, 2006). Even in the face of the M184V mutation, which is the mutation selecting for 3TC and FTC, data reflects that it is better to treat with either agent as monotherapy then to hold all HAART.

IV. Conclusion

HIV therapy has made tremendous advances in the last decade. The newer medicines are more effective, easier to take, and have fewer side effects than in the late 1990s. Although HIV is a rapidly adaptive virus, the new drugs are powerful weapons. In the United States where most patients have access to antiretroviral therapies, the only remaining stumbling block for patients is nonadherence. In essence, every HIV-infected individual in the United States should have an undetectable viral load. Unfortunately, many patients are still not fully adherent and, therefore, progress toward immunodeficiency.

References

Anonymous (2006). Guidelines for the use of antiretroviral agents in HIV-1 infected adults and adolescents. *In:* U.D.o.H.a.H. Services (Ed).

Anonymous (2007). ClinicalTrials.gov. *In:* N.I.o. Health (Ed).

Agenerase (2006). "Prescribing Information," GlaxoSmithKline, Research Triangle Park, NC.
Alvarez, D., Dieterich, D. T., Brau, N., Moorehead, L., Ball, L., and Sulkowski, M. S. (2006). Zidovudine use but not weight-based ribavirin dosing impacts anaemia during HCV treatment in HIV-infected persons. *J. Viral Hepat.* **13**(10), 683–689.
Aptivus (2005). "Prescribing Information," Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT.
Back, D., Gibbons, S., and Khoo, S. (2006). An update on therapeutic drug monitoring for antiretroviral drugs. *Ther. Drug Monit.* **28**(3), 468–473.
Balani, S. K., Kauffman, L. R., deLuna, F. A., and Lin, J. H. (1999). Nonlinear pharmacokinetics of efavirenz (DMP-266), a potent HIV-1 reverse transcriptase inhibitor, in rats and monkeys. *Drug Metab. Dispos.* **27**(1), 41–45.
Baldwin, J. R., Boron, M. T., Wang, Y., Schneck, D. W., and Hopkins, N. K. (1998). Effects of food and antacid on bioavailability of the protease inhibitor PNU-140690 in healthy volunteers. *Abstracts of the 5th Conference on Retroviruses and Opportunistic Infections*, Abstract 649.
Balzarini, J., Lee, C. K., Herdewijn, P., and De Clercq, E. (1991). Mechanism of the potentiating effect of ribavirin on the activity of 2′,3′-dideoxyinosine against human immunodeficiency virus. *J. Biol. Chem.* **266**(32), 21509–21514.
Barreiro, P., Rodríguez-Novoa, S., Labarga, P., Ruiz, A., Jiménez-Nácher, I., Martín-Carbonero, L., Gonzalez-Lahoz, J., and Soriano, V. (2007). Influence of liver fibrosis stage on plasma levels of antiretroviral drugs in HIV-infected patients with chronic hepatitis C. *J. Infect. Dis.* **195**(7), 973–979.
Behrens, G., Dejam, A., Schmidt, H., Balks, H. J., Brabant, G., Korner, T., Stoll, M., and Schmidt, R. E. (1999). Impaired glucose tolerance, beta cell function and lipid metabolism in HIV patients under treatment with protease inhibitors. *AIDS* **13**(10), F63–F70.
Bersoff-Matcha, S. J., Miller, W. C., Aberg, J. A., van Der Horst, C., Hamrick, H. J., Jr., Powderly, W. G., and Mundy, L. M. (2001). Sex differences in nevirapine rash. *Clin. Infect. Dis.* **32**(1), 124–129.
Blum, M. R., Liao, S. H., Good, S. S., and de Miranda, P. (1988). Pharmacokinetics and bioavailability of zidovudine in humans. *Am. J. Med.* **85**(2A), 189–194.
Boelaert, J. R., Dom, G. M., Huitema, A. D., Beijnen, J. H., and Lange, J. M. (2002). The boosting of didanosine by allopurinol permits a halving of the didanosine dosage. *AIDS* **16**(16), 2221–2223.
Boffito, M., Acosta, E., Burger, D., Fletcher, C. V., Flexner, C., Garaffo, R., Gatti, G., Kurowski, M., Perno, C. F., Peytavin, G., Regazzi, M., and Back, D. (2005). Current status and future prospects of therapeutic drug monitoring and applied clinical pharmacology in antiretroviral therapy. *Antivir. Ther.* **10**(3), 375–392.
Brinkman, K., ter Hofstede, H. J., Burger, D. M., Smeitink, J. A., and Koopmans, P. P. (1998). Adverse effects of reverse transcriptase inhibitors: Mitochondrial toxicity as common pathway. *AIDS* **12**(14), 1735–1744.
Brothers, C., Wannamaker, P., Sutherland-Phillips, D., and Hernandez, J. (2006). Lower reported rate of suspected hypersensitivity reaction (HSR) to abacavir (ABC) among black patients. Program and abstracts of the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, Abstract H-1065.
Browne, M. J., Mayer, K. H., Chafee, S. B., Dudley, M. N., Posner, M. R., Steinberg, S. M., Graham, K. K., Geletko, S. M., Zinner, S. H., Denman, S. L., *et al.* (1993). 2′,3′,-didehydro-3′,-deoxythymidine (d4T) in patients with AIDS or AIDS-related complex: A phase I trial. *J. Infect. Dis.* **167**(1), 21–29.
Burger, D. M., Hugen, P. W., Kroon, F. P., Groeneveld, P., Brinkman, K., Foudraine, N. A., Sprenger, H., Koopmans, P. P., and Hekster, Y. A. (1998). Pharmacokinetic interaction between the proton pump inhibitor omeprazole and the HIV protease inhibitor indinavir. *AIDS* **12**(15), 2080–2082.

Cadman, J. (1998). Efavirenz pregnancy warning. *GMHC Treat. Issues* **12**(3), 12.
Campbell, T. B., Shulman, N. S., Johnson, S. C., Zolopa, A. R., Young, R. K., Bushman, L., Fletcher, C. V., Lanier, E. R., Merigan, T. C., and Kuritzkes, D. R. (2005). Antiviral activity of lamivudine in salvage therapy for multidrug-resistant HIV-1 infection. *Clin. Infect. Dis.* **41**(2), 236–242.
Carr, A., Samaras, K., Burton, S., Law, M., Freund, J., Chisholm, D. J., and Cooper, D. A. (1998). A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* **12**(7), F51–F58.
Castagna, A., Danise, A., Menzo, S., Galli, L., Gianotti, N., Carini, E., Boeri, E., Galli, A., Cernuschi, M., Hasson, H., Clementi, M., and Lazzarin, A. (2006). Lamivudine monotherapy in HIV-1-infected patients harbouring a lamivudine-resistant virus: A randomized pilot study (E-184V study). *AIDS* **20**(6), 795–803.
Catanzaro, L. M., Slish, J. C., DiCenzo, R., and Morse, G. D. (2004). Drug interactions with antiretrovirals. *Curr. HIV/AIDS Rep.* **1**(2), 89–96.
Cheeseman, S. H., Hattox, S. E., McLaughlin, M. M., Koup, R. A., Andrews, C., Bova, C. A., Pav, J. W., Roy, T., Sullivan, J. L., and Keirns, J. J. (1993). Pharmacokinetics of nevirapine: Initial single-rising-dose study in humans. *Antimicrob. Agents Chemother.* **37**(2), 178–182.
Chittick, G. E., Gillotin, C., McDowell, J. A., Lou, Y., Edwards, K. D., Prince, W. T., and Stein, D. S. (1999). Abacavir: Absolute bioavailability, bioequivalence of three oral formulations, and effect of food. *Pharmacotherapy* **19**(8), 932–942.
Chun, T. W., Stuyver, L., Mizell, S. B., Ehler, L. A., Mican, J. A., Baseler, M., Lloyd, A. L., Nowak, M. A., and Fauci, A. S. (1997). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* **94**(24), 13193–13197.
Companies, A.s.P. (2006). Medicines in development for HIV/AIDS.
Cooper, D., Steinhart, C., and Ward, D. (2006). Efficacy and safety results at 48 weeks with the novel NNRTI, TMC125, and impact of baseline resistance on the virologic response in study TMC125-C223. *XVI International AIDS Conference*, Abstract TUPE0061.
Crixivan (2006). "Prescribing Information," Merck & Co, Inc., Whitehouse Station, NJ.
Crowe, S., Cooper, D. A., and Chambers, D. (1996). Antiviral therapies for HIV: Managing HIV. *Med. J. Aust.* **164**, 290–295.
Cundy, K. C., Sueoka, C., Lynch, G. R., Griffin, L., Lee, W. A., and Shaw, J. P. (1998). Pharmacokinetics and bioavailability of the anti-human immunodeficiency virus nucleotide analog 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in dogs. *Antimicrob. Agents Chemother.* **42**(3), 687–690.
D'Aquila, R. T., Hughes, M. D., Johnson, V. A., Fischl, M. A., Sommadossi, J. P., Liou, S. H., Timpone, J., Myers, M., Basgoz, N., Niu, M., and Hirsch, M. S. (1996). Nevirapine, zidovudine, and didanosine compared with zidovudine and didanosine in patients with HIV-1 infection. A randomized, double-blind, placebo-controlled trial. national institute of allergy and infectious diseases AIDS clinical trials group protocol 241 investigators. *Ann. Intern. Med.* **124**(12), 1019–1030.
Davey, R. T., Jr., Chaitt, D. G., Reed, G. F., Freimuth, W. W., Herpin, B. R., Metcalf, J. A., Eastman, P. S., Falloon, J., Kovacs, J. A., Polis, M. A., Walker, R. E., and Masur, H. (1996). Randomized, controlled phase I/II, trial of combination therapy with delavirdine (U-90152S) and conventional nucleosides in human immunodeficiency virus type 1-infected patients. *Antimicrob. Agents Chemother.* **40**(7), 1657–1664.
De Clercq, E. (1995). Antiviral therapy for human immunodeficiency virus infections. *Clin. Microbiol. Rev.* **8**(2), 200–239.
Deeks, S. G. (1998). Antiretroviral agents: The next generation. *AIDS Clin. Care* **10**(5), 33–36, 39–40.
Deeks, S. G., and Volberding, P. A. (1997). HIV-1 protease inhibitors. *AIDS Clin. Rev.* 145–185.
DeJesus, E., Herrera, G., Teofilo, E., Gerstoft, J., Buendia, C. B., Brand, J. D., Brothers, C. H., Hernandez, J., Castillo, S. A., Bonny, T., Lanier, E. R., and Scott, T. R. (2004). Abacavir

versus zidovudine combined with lamivudine and efavirenz, for the treatment of antiretroviral-naive HIV-infected adults. *Clin. Infect. Dis.* **39**(7), 1038–1046.

Delfraissy, J.-F., Flandre, P., and Delaugerre, C. (2006). MONARK trial: 48-week analysis of lopinavir/ritonavir (LPV/r) monotherapy compated to LPV/r + zidovudine/lamivudine in antiretroviral-naive patients. International AIDS Conference.

Dolin, R., Amato, D. A., Fischl, M. A., Pettinelli, C., Beltangady, M., Liou, S. H., Brown, M. J., Cross, A. P., Hirsch, M. S., Hardy, W. D., *et al.* (1995). Zidovudine compared with didanosine in patients with advanced HIV type 1 infection and little or no previous experience with zidovudine. AIDS clinical trials group. *Arch. Intern. Med.* **155**(9), 961–974.

Dudley, M. N., Graham, K. K., Kaul, S., Geletko, S., Dunkle, L., Browne, M., and Mayer, K. (1992). Pharmacokinetics of stavudine in patients with AIDS or AIDS-related complex. *J. Infect. Dis.* **166**(3), 480–485.

Easterbrook, P. J., Waters, A., Murad, S., Ives, N., Taylor, C., King, D., Vyakarnam, A., and Thorburn, D. (2003). Epidemiological risk factors for hypersensitivity reactions to abacavir. *HIV Med.* **4**(4), 321–324.

El-Sadr, W. M., Lundgren, J. D., Neaton, J. D., Gordin, F., Abrams, D., Arduino, R. C., Babiker, A., Burman, W., Clumeck, N., Cohen, C. J., Cohn, D., and Cooper, D. (2006). CD4 + count-guided interruption of antiretroviral treatment. *N. Engl. J. Med.* **355**(22), 2283–2296.

Emtriva (2006). "Prescribing Information," Gilead Sciences, Inc., Foster City, CA.

Epivir (2006). "Prescribing Information," GlaxoSmithKline, Research Triangle Park, NC.

Eron, J., Jr., Yeni, P., Gathe, J., Jr., Estrada, V., DeJesus, E., Staszewski, S., Lackey, P., Katlama, C., Young, B., Yau, L., Sutherland-Phillips, D., Wannamaker, P., *et al.* (2006). The KLEAN study of fosamprenavir-ritonavir versus lopinavir-ritonavir, each in combination with abacavir-lamivudine, for initial treatment of HIV infection over 48 weeks: A randomised non-inferiority trial. *Lancet* **368**(9534), 476–482.

Eron, J. J., Benoit, S. L., Jemsek, J., MacArthur, R. D., Santana, J., Quinn, J. B., Kuritzkes, D. R., Fallon, M. A., and Rubin, M. (1995). Treatment with lamivudine, zidovudine, or both in HIV-positive patients with 200 to 500 CD4 + cells per cubic millimeter. North American HIV working party. *N. Engl. J. Med.* **333**(25), 1662–1669.

Falloon, J., Piscitelli, S., Vogel, S., Sadler, B., Mitsuya, H., Kavlick, M. F., Yoshimura, K., Rogers, M., LaFon, S., Manion, D. J., Lane, H. C., and Masur, H. (2000). Combination therapy with amprenavir, abacavir, and efavirenz in human immunodeficiency virus (HIV)-infected patients failing a protease-inhibitor regimen: Pharmacokinetic drug interactions and antiviral activity. *Clin. Infect. Dis.* **30**(2), 313–318.

Farthing, C., Khanlou, H., and Yeh, V. (2003). Early virologic failure in a pilot study evaluating the efficacy of abacavir, lamivudine and tenofovir in the treatment naive HIV-infected patients. International AIDS Society Conference on HIV Pathogenesis and Treatment.

Ferrari, A., Coccia, C. P., Bertolini, A., and Sternieri, E. (2004). Methadone–metabolism, pharmacokinetics and interactions. *Pharmacol. Res.* **50**(6), 551–559.

Fletcher, C. (2006). Navigating the concentration curves: Pharmacologic considerations in the treatment of HIV. *Adv. Stud. Med.* **6**(9B), S853–S872.

Foster, R. H., and Faulds, D. (1998). Abacavir. *Drugs* **55**(5), 729–736: discussion 737–738.

Freimuth, W. W., Chuang-Stein, C. J., Greenwald, C. A., Cox, S. R., Edge-Padbury, B. A., Carberry, P. A., and Wathen, L. K. (1996). Delavirdine (DLV) + didanosine (ddI) combination therapy has sustained surrogate marker response in advanced HIV-1 population (abstract). *3rd Conf Retro Opportun Infect*, Abstract 163.

Fung, H. B., Stone, E. A., and Piacenti, F. J. (2002). Tenofovir disoproxil fumarate: A nucleotide reverse transcriptase inhibitor for the treatment of HIV infection. *Clin. Ther.* **24**(10), 1515–1548.

Fuzeon (2004). "Prescribing Information," Roche Laboratories, Nutley, NJ.

Gallant, J. E., DeJesus, E., Arribas, J. R., Pozniak, A. L., Gazzard, B., Campo, R. E., Lu, B., McColl, D., Chuck, S., Enejosa, J., Toole, J. J., and Cheng, A. K. (2006). Tenofovir DF,

emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. *N. Engl. J. Med.* **354**(3), 251–260.

Gao, W. Y., Shirasaka, T., Johns, D. G., Broder, S., and Mitsuya, H. (1993). Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. *J. Clin. Invest.* **91**(5), 2326–2333.

Gathe, J. C., Jr., Ive, P., Wood, R., Schurmann, D., Bellos, N. C., DeJesus, E., Gladysz, A., Garris, C., and Yeo, J. (2004). SOLO: 48-week efficacy and safety comparison of once-daily fosamprenavir /ritonavir versus twice-daily nelfinavir in naive HIV-1-infected patients. *AIDS* **18**(11), 1529–1537.

Greeson, J. M., Sanford, B., and Monti, D. A. (2001). St. John's wort (Hypericum perforatum): A review of the current pharmacological, toxicological, and clinical literature. *Psychopharmacology (Berl.)* **153**(4), 402–414.

Grobler, J. A., Stillmock, K., Hu, B., Witmer, M., Felock, P., Espeseth, A. S., Wolfe, A., Egbertson, M., Bourgeois, M., Melamed, J., Wai, J. S., Young, S., *et al.* (2002). Diketo acid inhibitor mechanism and HIV-1 integrase: Implications for metal binding in the active site of phosphotransferase enzymes. *Proc. Natl. Acad. Sci. USA* **99**(10), 6661–6666.

Gulick, R. M., Ribaudo, H. J., Shikuma, C. M., Lustgarten, S., Squires, K. E., Meyer, W. A., III, Acosta, E. P., Schackman, B. R., Pilcher, C. D., Murphy, R. L., Maher, W. E., Witt, M. D., *et al.* (2004). Triple-nucleoside regimens versus efavirenz-containing regimens for the initial treatment of HIV-1 infection. *N. Engl. J. Med.* **350**(18), 1850–1861.

Haas, D. W., Smeaton, L. M., Shafer, R. W., Robbins, G. K., Morse, G. D., Labbe, L., Wilkinson, G. R., Clifford, D. B., D'Aquila, R. T., De Gruttola, V., Pollard, R. B., Merigan, T. C., *et al.* (2005). Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: An adult aids clinical trials group study. *J. Infect. Dis.* **192**(11), 1931–1942.

Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., Eron, J. J., Jr., Feinberg, J. E., Balfour, H. H., Jr., Deyton, L. R., Chodakewitz, J. A., and Fischl, M. A. (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS clinical trials group 320 study team. *N. Engl. J. Med.* **337**(11), 725–733.

Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* **287**(5453), 646–650.

Hicks, C., DeJesus, E., Sloan, L., *et al.* (2006). Efficacy and safety of once-daily boosted fosamprenavir/ritonavir with abacavir/lamivudine fixed dose combination in antiretroviral naive HIV-1 infected patients: 24-week results from COL100758. *8th International Congress on Drug Therapy in HIV Infection*, Abstract P2.

Hirani, V. N., Raucy, J. L., and Lasker, J. M. (2004). Conversion of the HIV protease inhibitor nelfinavir to a bioactive metabolite by human liver CYP2C19. *Drug Metab. Dispos.* **32**(12), 1462–1467.

Hoetelmans, R. M., van Heeswijk, R. P., Profijt, M., Mulder, J. W., Meenhorst, P. L., Lange, J. M., Reiss, P., and Beijnen, J. H. (1998). Comparison of the plasma pharmacokinetics and renal clearance of didanosine during once and twice daily dosing in HIV-1 infected individuals. *AIDS* **12**(17), F211–F216.

Hoggard, P. G., Veal, G. J., Wild, M. J., Barry, M. G., and Back, D. J. (1995). Drug interactions with zidovudine phosphorylation in vitro. *Antimicrob. Agents Chemother.* **39**(6), 1376–1378.

Hoggard, P. G., Kewn, S., Barry, M. G., Khoo, S. H., and Back, D. J. (1997). Effects of drugs on 2′,3′-dideoxy-2′,3′-didehydrothymidine phosphorylation in vitro. *Antimicrob. Agents Chemother.* **41**(6), 1231–1236.

Honda, M., Yasuoka, A., Aoki, M., and Oka, S. (1999). A generalized seizure following initiation of nelfinavir in a patient with human immunodeficiency virus type 1 infection, suspected due to interaction between nelfinavir and phenytoin. *Intern. Med.* **38**(3), 302–303.

Huisman, M. T., Smit, J. W., and Schinkel, A. H. (2000). Significance of P-glycoprotein for the pharmacology and clinical use of HIV protease inhibitors. *AIDS* **14**(3), 237–242.

Invirase (2005). "Prescribing Information," Roche Laboratories, Nutley, NJ.

Jensen-Fangel, S., Justesen, U. S., Black, F. T., Pedersen, C., and Obel, N. (2003). The use of calcium carbonate in nelfinavir-associated diarrhoea in HIV-1-infected patients. *HIV Med.* **4**(1), 48–52.

Johnson, M., Grinsztejn, B., Rodriguez, C., Coco, J., DeJesus, E., Lazzarin, A., Lichtenstein, K., Rightmire, A., Sankoh, S., and Wilber, R. (2005). Atazanavir plus ritonavir or saquinavir, and lopinavir/ritonavir in patients experiencing multiple virological failures. *AIDS* **19**(7), 685–694.

Johnson, M. A., Gathe, J. C., Jr., Podzamczer, D., Molina, J. M., Naylor, C. T., Chiu, Y. L., King, M. S., Podsadecki, T. J., Hanna, G. J., and Brun, S. C. (2006). A once-daily lopinavir/ritonavir-based regimen provides noninferior antiviral activity compared with a twice-daily regimen. *J. Acquir. Immune Defic. Syndr.* **43**(2), 153–160.

Kahn, J. O., Lagakos, S. W., Richman, D. D., Cross, A., Pettinelli, C., Liou, S. H., Brown, M., Volberding, P. A., Crumpacker, C. S., Beall, G., *et al.* (1992). A controlled trial comparing continued zidovudine with didanosine in human immunodeficiency virus infection. The NIAID AIDS clinical trials group. *N. Engl. J. Med.* **327**(9), 581–587.

Kaletra (2006). "Prescribing Information," Abbott Laboratories, North Chicago, IL.

Kaul, S., Bassi, K., Damle, B., Xie, J., Gale, J., Kearney, B., and Hanna, G. (2003). Pharmacokinetic (PK) evaluation of the combination of atazanavir (ATV), the enteric coated didanosine (ddI-EC), and tenofovir disoproxil fumarate (TDF) for a once-daily antiretroviral. *Program and abstracts at the 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abstract A-1616.

Kearney, B., Flaherty, J., Wolf, F., *et al.* (2001). Lack of clinically relevant drug-drug interactions between tenofovir DF and efavirenz, indinavir, lamivudine and lopinavir/ritonavir in healthy subjects. *Presented at: 8th European Conference on Clinical Aspects and Treatment of HIV Infection.*

Kilby, J. M., and Eron, J. J. (2003). Novel therapies based on mechanisms of HIV-1 cell entry. *N. Engl. J. Med.* **348**(22), 2228–2238.

Kilby, J. M., Hill, A., and Buss, N. (2002). The effect of ritonavir on saquinavir plasma concentration is independent of ritonavir dosage: Combined analysis of pharmacokinetic data from 97 subjects. *HIV Med.* **3**(2), 97–104.

Kopp, J. B., Miller, K. D., Mican, J. A., Feuerstein, I. M., Vaughan, E., Baker, C., Pannell, L. K., and Falloon, J. (1997). Crystalluria and urinary tract abnormalities associated with indinavir. *Ann. Intern. Med.* **127**(2), 119–125.

Krikorian, S. A., and Rudorf, D. C. (2005). Drug-drug interactions and HIV therapy: What should pharmacist know? *J. Pharm. Pract.* **18**, 278–294.

la Porte, C. J., de Graaff-Teulen, M. J., Colbers, E. P., Voncken, D. S., Ibanez, S. M., Koopmans, P. P., Hekster, Y. A., and Burger, D. M. (2004). Effect of efavirenz treatment on the pharmacokinetics of nelfinavir boosted by ritonavir in healthy volunteers. *Br. J. Clin. Pharmacol.* **58**(6), 632–640.

Lambert, J. S., Seidlin, M., Reichman, R. C., Plank, C. S., Laverty, M., Morse, G. D., Knupp, C., McLaren, C., Pettinelli, C., Valentine, F. T., *et al.* (1990). 2′,3′-dideoxyinosine (ddI) in patients with the acquired immunodeficiency syndrome or AIDS-related complex. A phase I trial. *N. Engl. J. Med.* **322**(19), 1333–1340.

Lee, W. A., and Martin, J. C. (2006). Perspectives on the development of acyclic nucleotide analogs as antiviral drugs. *Antiviral Res.* **71**(2–3), 254–259.

Lertora, J. J., Rege, A. B., Greenspan, D. L., Akula, S., George, W. J., Hyslop, N. E., Jr., and Agrawal, K. C. (1994). Pharmacokinetic interaction between zidovudine and valproic acid in patients infected with human immunodeficiency virus. *Clin. Pharmacol. Ther.* **56**(3), 272–278.

Liedtke, M. D., Lockhart, S. M., and Rathbun, R. C. (2004). Anticonvulsant and antiretroviral interactions. *Ann. Pharmacother.* **38**(3), 482–489.

Lonergan, J. T., Behling, C., Pfander, H., Hassanein, T. I., and Mathews, W. C. (2000). Hyperlactatemia and hepatic abnormalities in 10 human immunodeficiency virus-infected patients receiving nucleoside analogue combination regimens. *Clin. Infect. Dis.* **31**(1), 162–166.

MacArthur, R. D., Novak, R. M., Peng, G., Chen, L., Xiang, Y., Hullsiek, K. H., Kozal, M. J., van den Berg-Wolf, M., Henely, C., Schmetter, B., and Dehlinger, M. (2006). A comparison of three highly active antiretroviral treatment strategies consisting of non-nucleoside reverse transcriptase inhibitors, protease inhibitors, or both in the presence of nucleoside reverse transcriptase inhibitors as initial therapy (CPCRA 058 FIRST Study): A long-term randomised trial. *Lancet* **368**(9553), 2125–2135.

Malik, A., Abraham, P., and Malik, N. (2005). Acute renal failure and Fanconi syndrome in an AIDS patient on tenofovir treatment–case report and review of literature. *J. Infect.* **51**(2), E61–E65.

Mallal, S., Nolan, D., Witt, C., Masel, G., Martin, A. M., Moore, C., Sayer, D., Castley, A., Mamotte, C., Maxwell, D., James, I., and Christiansen, F. T. (2002). Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet* **359**(9308), 727–732.

Markowitz, J. S., Donovan, J. L., DeVane, C. L., Taylor, R. M., Ruan, Y., Wang, J. S., and Chavin, K. D. (2003). Effect of St John's wort on drug metabolism by induction of cytochrome P450 3A4 enzyme. *Jama* **290**(11), 1500–1504.

Markowitz, M., Conant, M., Hurley, A., Schluger, R., Duran, M., Peterkin, J., Chapman, S., Patick, A., Hendricks, A., Yuen, G. J., Hoskins, W., Clendeninn, N., and Ho, D. D. (1998). A preliminary evaluation of nelfinavir mesylate, an inhibitor of human immunodeficiency virus (HIV)-1 protease, to treat HIV infection. *J. Infect. Dis.* **177**(6), 1533–1540.

McDonald, C. K., and Kuritzkes, D. R. (1997). Human immunodeficiency virus type 1 protease inhibitors. *Arch. Intern. Med.* **157**(9), 951–959.

McDowell, J. A., Chittick, G. E., Stevens, C. P., Edwards, K. D., and Stein, D. S. (2000). Pharmacokinetic interaction of abacavir (1592U89) and ethanol in human immunodeficiency virus-infected adults. *Antimicrob. Agents Chemother.* **44**(6), 1686–1690.

Merry, C., Barry, M. G., Mulcahy, F., Ryan, M., Heavey, J., Tjia, J. F., Gibbons, S. E., Breckenridge, A. M., and Back, D. J. (1997). Saquinavir pharmacokinetics alone and in combination with ritonavir in HIV-infected patients. *AIDS* **11**(4), F29–F33.

Miralles Alvarez, C., Torres Perea, R., SanzSanz, J., Ocaña, I., Hervás, R., Vergara, A., Lorenzo, A., Dalmau, D., Cervantes, M., Knobel, H., de la Fuente García, B., Flores Cid, J., *et al.* (2004). Identification and follow up of adverse events related to NRTI use that in the clinical practice causes its substitution for tenofovir DF (RECOVER study). 15th International AIDS Conference.

Montaner, J. S., Reiss, P., Cooper, D., Vella, S., Harris, M., Conway, B., Wainberg, M. A., Smith, D., Robinson, P., Hall, D., Myers, M., and Lange, J. M. (1998). A randomized, double-blind trial comparing combinations of nevirapine, didanosine, and zidovudine for HIV-infected patients: The INCAS Trial. Italy, the Netherlands, Canada and Australia study. *Jama* **279**(12), 930–937.

Moore, K. H., Yuen, G. J., Raasch, R. H., Eron, J. J., Martin, D., Mydlow, P. K., and Hussey, E. K. (1996). Pharmacokinetics of lamivudine administered alone and with trimethoprim-sulfamethoxazole. *Clin. Pharmacol. Ther.* **59**(5), 550–558.

Moreno, A., Quereda, C., Moreno, L., Perez-Elías, M. J., Muriel, A., Casado, J. L., Antela, A., Dronda, F., Navas, E., Bárcena, R., and Moreno, S. (2004). High rate of didanosine-related mitochondrial toxicity in HIV/HCV-coinfected patients receiving ribavirin. *Antiviral Ther.* **9**(1), 133–138.

Morse, C. G., and Kovacs, J. A. (2006). Metabolic and skeletal complications of HIV infection: The price of success. *Jama* **296**(7), 844–854.

Murata, H., Hruz, P. W., and Mueckler, M. (2000). The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *J. Biol. Chem.* **275**(27), 20251–20254.

Negredo, E., Molto, J., Burger, D., Viciana, P., Ribera, E., Paredes, R., Juan, M., Ruiz, L., Puig, J., Pruvost, A., Grassi, J., Masmitja, E., *et al.* (2004). Unexpected CD4 cell count decline in patients receiving didanosine and tenofovir-based regimens despite undetectable viral load. *AIDS* **18**(3), 459–463.

Norvir (2006). "Prescribing Information," Abbott Laboratories, North Chicago, IL.

Phillips, A. (2004). Short-term risk of AIDS according to current CD4 cell count and viral load in antiretroviral drug-naive individuals and those treated in the monotherapy era. *AIDS* **18**(1), 51–58.

Podzamczer, D., Ferrer, E., Sanchez, P., Gatell, J. M., Crespo, M., Fisac, C., Lonca, M., Sanz, J., Niubo, J., Veloso, S., Llibre, J. M., Barrufet, P., *et al.* (2006). Less lipoatrophy and better lipid profile with abacavir as compared to stavudine: 96-Week results of a randomized study. *J. Acquir. Immune Defic. Syndr.* **44**(2), 139–147.

Poppe, S. M., Slade, D. E., Chong, K. T., Hinshaw, R. R., Pagano, P. J., Markowitz, M., Ho, D. D., Mo, H., Gorman, R. R., III, Dueweke, T. J., Thaisrivongs, S., and Tarpley, W. G. (1997). Antiviral activity of the dihydropyrone PNU-140690, a new nonpeptidic human immunodeficiency virus protease inhibitor. *Antimicrob. Agents Chemother.* **41**(5), 1058–1063.

Prezista (2006). "Prescribing Information," Tibotec Therapeutics, Raritan, NJ.

Quart, B. D., Chapman, S. K., Peterkin, J., Webber, S., and Oliver, S. (1995). Phase I safety, tolerance, pharmacokinetics and food effect studies of AG1343-a novel HIV protease inhibitor. *Second National Conference on Human Retroviruses and Related Infections*, Abstract LB3.

Ray, A. S., Olson, L., and Fridland, A. (2004). Role of purine nucleoside phosphorylase in interactions between 2′,3′-dideoxyinosine and allopurinol, ganciclovir, or tenofovir. *Antimicrob. Agents Chemother.* **48**(4), 1089–1095.

Reeves, I., Fishaer, M., and Churchill, D. (2006). Clinical utility of HLA-B*5701 testing in a UK clinic cohort. *Program and abstracts of the 13th Conference on Retroviruses and Opportunistic Infections*, Abstract 667a.

Rescriptor (2006). "Prescribing Information," Pharmacia & Upjohn Company, Kalamazoo, MI.

Retrovir (2006). "Prescribing Information," GlaxoSmithKline, Research Triangle Park, NC.

Reyataz (2006). "Prescribing Information," Bristol-Myers Squibb, Princeton, NJ.

Richman, D. D., Fischl, M. A., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D., Hirsch, M. S., *et al.* (1987). The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *N. Engl. J. Med.* **317**(4), 192–197.

Riddler, S. A., Haubrich, R., DiRienzo, G., Peeples, L., Powderly, W. G., Klingman, K. L., Garren, K. W., George, T., Rooney, J. F., Brizz, B., Havlir, D., Mellors, J. W., *et al.* (2006). A prospective, randomized phase III trial of NRTI-, PI- and NNRTI-sparing regimens for initial treatment of HIV-1 infection-ACTG 5142. International AIDS Conference.

Robbins, G. K., De Gruttola, V., Shafer, R. W., Smeaton, L. M., Snyder, S. W., Pettinelli, C., Dube, M. P., Fischl, M. A., Pollard, R. B., Delapenha, R., Gedeon, L., van der Horst, C., *et al.* (2003). Comparison of sequential three-drug regimens as initial therapy for HIV-1 infection. *N. Engl. J. Med.* **349**(24), 2293–2303.

Rodriguez-Torres, M., Torriani, F. J., Soriano, V., Borucki, M. J., Lissen, E., Sulkowski, M., Dieterich, D., Wang, K., Gries, J. M., Hoggard, P. G., and Back, D. (2005). Effect of ribavirin on intracellular and plasma pharmacokinetics of nucleoside reverse transcriptase inhibitors in patients with human immunodeficiency virus-hepatitis C virus coinfection: Results of a randomized clinical study. *Antimicrob. Agents Chemother.* **49**(10), 3997–4008.

Roszko, P. J., Curry, K., Brazina, B., Cohen, A., Turkie, E. L., Sabo, J. P., MacGregor, T. R., and McCallister, S. (2003). Standard doses of efavirenz (EFV), zidovudine (ZDV), tenofovir (TDF), and didanosine (ddI) may be given with tipranavir/ritonavir (TPV/r). *International AIDS Society 2nd Conferene of Pathogenesis and Treatment*, Abstract No. 865.

Ruane, P., Luber, A., Wire, M. B., *et al.* (2004). Plasma amprenavir pharmacokinetics and safety following co-administration of fosamprenavir with a reduced ritonavir dose once daily (COL 10053). *Program and abstracts of the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abstract A-449.

Saint-Marc, T., and Touraine, J. L. (1999). The effects of discontinuing stavudine therapy on clinical and metabolic abnormalities in patients suffering from lipodystrophy. *AIDS* **13**(15), 2188–2189.

Schaaf, B., Aries, S. P., Kramme, E., Steinhoff, J., and Dalhoff, K. (2003). Acute renal failure associated with tenofovir treatment in a patient with acquired immunodeficiency syndrome. *Clin. Infect. Dis.* **37**(3), e41–e43.

Schewe, C. K., Maserati, R., Wassmer, G., Adam, A., and Weitner, L. (2006). Improved lipid profiles and maintenance of virologic control in heavily pretreated HIV-infected patients who switched from stavudine to tenofovir treatment. *Clin. Infect. Dis.* **42**(1), 145–147.

Scott, L. J., and Perry, C. M. (2000). Delavirdine: A review of its use in HIV infection. *Drugs* **60**(6), 1411–1444.

Sham, H. L., Kempf, D. J., Molla, A., Marsh, K. C., Kumar, G. N., Chen, C. M., Kati, W., Stewart, K., Lal, R., Hsu, A., Betebenner, D., Korneyeva, M., *et al.* (1998). ABT-378, a highly potent inhibitor of the human immunodeficiency virus protease. *Antimicrob. Agents Chemother.* **42**(12), 3218–3224.

Solas, C., Poizot-Martin, I., Drogoul, M. P., Ravaux, I., Dhiver, C., Lafeuillade, A., Allegre, T., Mokhtari, M., Moreau, J., Lepeu, G., Petit, N., Durand, A., *et al.* (2004). Therapeutic drug monitoring of lopinavir/ritonavir given alone or with a non-nucleoside reverse transcriptase inhibitor. *Br. J. Clin. Pharmacol.* **57**(4), 436–440.

Sosa, N., Hill-Zabala, C., Dejesus, E., Herrera, G., Florance, A., Watson, M., Vavro, C., and Shaefer, M. (2005). Abacavir and lamivudine fixed-dose combination tablet once daily compared with abacavir and lamivudine twice daily in HIV-infected patients over 48 weeks (ESS30008, SEAL). *J. Acquir. Immune Defic. Syndr.* **40**(4), 422–427.

Squires, K., Lazzarin, A., Gatell, J. M., Powderly, W. G., Pokrovskiy, V., Delfraissy, J. F., Jemsek, J., Rivero, A., Rozenbaum, W., Schrader, S., Sension, M., and Vibhagool, A. (2004). Comparison of once-daily atazanavir with efavirenz, each in combination with fixed-dose zidovudine and lamivudine, as initial therapy for patients infected with HIV. *J. Acquir. Immune Defic. Syndr.* **36**(5), 1011–1019.

Sustiva (2006). "Prescribing Information," Bristol-Myers Squibb, Princeton, NJ.

Swindells, S., Wilkin, T., DiRienzo, G., *et al.* (2006). A prospective, open-label trial of regimen simplifications to atazanavir/ritonavir alone as maintenance antiretroviral therapy after sustained virologic suppression (ACTG 5201). Conference on Retroviruses and Opportunistic Infection.

Taburet, A. M., Naveau, S., Zorza, G., Colin, J. N., Delfraissy, J. F., Chaput, J. C., and Singlas, E. (1990). Pharmacokinetics of zidovudine in patients with liver cirrhosis. *Clin. Pharmacol. Ther.* **47**(6), 731–739.

Taburet, A. M., Piketty, C., Chazallon, C., Vincent, I., Gerard, L., Calvez, V., Clavel, F., Aboulker, J. P., and Girard, P. M. (2004). Interactions between atazanavir-ritonavir and

tenofovir in heavily pretreated human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **48**(6), 2091–2096.

Tashima, K. T., Bausserman, L., Alt, E. N., Aznar, E., and Flanigan, T. P. (2003). Lipid changes in patients initiating efavirenz- and indinavir-based antiretroviral regimens. *HIV Clin. Trials* **4**(1), 29–36.

Tavassoli, N., Bagheri, H., Sommet, A., Delpierre, C., Marion-Latard, F., Massip, P., Aquilina, C., Bonnet, E., Obadia, M., Labau, E., Montastruc, J. L., and Bernard, J. (2006). Effects of discontinuing stavudine or protease inhibitor therapy on human immunodeficiency virus-related fat redistribution evaluated by dual-energy x-ray absorptiometry. *Pharmacotherapy* **26**(2), 154–161.

Tebas, P., Powderly, W. G., Claxton, S., Marin, D., Tantisiriwat, W., Teitelbaum, S. L., and Yarasheski, K. E. (2000). Accelerated bone mineral loss in HIV-infected patients receiving potent antiretroviral therapy. *AIDS* **14**(4), F63–F67.

van Leeuwen, R., Lange, J. M., Hussey, E. K., Donn, K. H., Hall, S. T., Harker, A. J., Jonker, P., and Danner, S. A. (1992). The safety and pharmacokinetics of a reverse transcriptase inhibitor, 3TC, in patients with HIV infection: A phase I study. *AIDS* **6**(12), 1471–1475.

van Leth, F., Hassink, E., Phanuphak, P., Miller, S., Gazzard, B., Cahn, P., Wood, R., Squires, K., Katlama, C., Santos, B., Robinson, P., van Leeuwen, R., *et al.* (2003). Results of the 2NN study: A randomized comparative trial of first-line antiretroviral therapy with regimens containing either nevirapine alone, efavirenz alone or both drugs combined, together with stavudine and lamivudine. Conference on Retroviruses and Opportunistic Infections.

van Leth, F., Phanuphak, P., Ruxrungtham, K., Baraldi, E., Miller, S., Gazzard, B., Cahn, P., Lalloo, U. G., van der Westhuizen, I. P., Malan, D. R., Johnson, M. A., Santos, B. R., *et al.* (2004). Comparison of first-line antiretroviral therapy with regimens including nevirapine, efavirenz, or both drugs, plus stavudine and lamivudine: A randomised open-label trial, the 2NN Study. *Lancet* **363**(9417), 1253–1263.

Vfend (2006). "Prescribing Information," Roerig, Division of Pfizer, Inc., New York, NY.

VidexEC (2006). "Prescribing Information," Bristol-Myers Squibb, Princeton, NJ.

Viracept (2006). "Prescribing Information," Agouron Pharmaceuticals, Inc., La Jolla, CA.

Viramune (2005). "Prescribing Information," Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT.

Viread (2006). "Prescribing Information," Gilead Sciences, Inc., Foster City, CA.

Walker, U. A., Setzer, B., and Venhoff, N. (2002). Increased long-term mitochondrial toxicity in combinations of nucleoside analogue reverse-transcriptase inhibitors. *AIDS* **16**(16), 2165–2173.

Walmsley, S., Bernstein, B., King, M., Arribas, J., Beall, G., Ruane, P., Johnson, M., Johnson, D., Lalonde, R., Japour, A., Brun, S., and Sun, E. (2002). Lopinavir-ritonavir versus nelfinavir for the initial treatment of HIV infection. *N. Engl. J. Med.* **346**(26), 2039–2046.

Walmsley, S., Leith, J., Katlama, C., Arasteh, K., Pierone, G., Blick, G., Lazzarin, A., Johnson, M., Samuels, C., Jones, P., Chaves, R., Quinson, A., *et al.* (2004). Pharmacokinetics and safety of Tipranavir/Ritonavir (TPV/r) alone or in combination with saquinavir (SQV), amprenavir (APV), or lopinavir (LPV): Interim analysis of BI 1182.51. *15th International AIDS Conference*, WeOrB1236.

Weinstock, H. S., Zaidi, I., Heneine, W., Bennett, D., Garcia-Lerma, J. G., Douglas, J. M., Jr., LaLota, M., Dickinson, G., Schwarcz, S., Torian, L., Wendell, D., Paul, S., *et al.* (2004). The epidemiology of antiretroviral drug resistance among drug-naive HIV-1-infected persons in 10 US cities. *J. Infect. Dis.* **189**(12), 2174–2180.

Wensing, A. M., van de Vijver, D. A., Angarano, G., Asjo, B., Balotta, C., Boeri, E., Camacho, R., Chaix, M. L., Costagliola, D., De Luca, A., Derdelinckx, I., Grossman, Z., *et al.* (2005). Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: Implications for clinical management. *J. Infect. Dis.* **192**(6), 958–966.

Yarchoan, R., Mitsuya, H., Myers, C. E., and Broder, S. (1989). Clinical pharmacology of 3′-azido-2′,3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. *N. Engl. J. Med.* **321**(11), 726–738.

Yeh, K. C., Deutsch, P. J., Haddix, H., Hesney, M., Hoagland, V., Ju, W. D., Justice, S. J., Osborne, B., Sterrett, A. T., Stone, J. A., Woolf, E., and Waldman, S. (1998). Single-dose pharmacokinetics of indinavir and the effect of food. *Antimicrob. Agents Chemother.* **42**(2), 332–338.

Yeni, P., Cooper, D. A., Aboulker, J. P., Babiker, A. G., Carey, D., Darbyshire, J. H., Floridia, M., Girard, P. M., Goodall, R. L., Hooker, M. H., Mijch, A., Meiffredy, V., *et al.* (2006). Virological and immunological outcomes at 3 years after starting antiretroviral therapy with regimens containing non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or both in INITIO: Open-label randomised trial. *Lancet* **368**(9532), 287–298.

Youle, M., Gerstoft, J., Fox, Z., *et al.* (2003). The final week 48 analysis of a phase IV, randomised, open-label, multi-centre trial to evaluate safety and efficacy of lopinavir/ritonavir (400/100 mg bid) versus saquinavir/ ritonavir (1000/100 mg bid): The MaxCmin2 trial. *Program and abstracts of the 9th European AIDS Conference (EACS), 1st EACS Resistance & Pharmacology Workshop*, Abstract F11/3.

Zerit (2006). "Prescribing Information," Bristol-Myers Squibb, Princeton, NJ.

Zhou, S., Chan, E., Pan, S. Q., Huang, M., and Lee, E. J. (2004). Pharmacokinetic interactions of drugs with St John's wort. *J. Psychopharmacol.* **18**(2), 262–276.

Zucker, S. D., Qin, X., Rouster, S. D., Yu, F., Green, R. M., Keshavan, P., Feinberg, J., and Sherman, K. E. (2001). Mechanism of indinavir-induced hyperbilirubinemia. *Proc. Natl. Acad. Sci. USA* **98**(22), 12671–12676.

This page intentionally left blank

Alexandre Harari and Giuseppe Pantaleo

Laboratory of AIDS Immunopathogenesis, Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland

HIV-1-Specific Immune Response

I. Introduction

The innate immune system represents the first line of defense against invading microorganisms, and there is increasing evidence that the initial interaction between the invading microorganisms and the innate immune system substantially shapes the development of the adaptive immunity. However, protection against virus infections is predominantly mediated by adaptive immunity and by both humoral and cell-mediated immunity. The antiviral effect of humoral immunity is mediated through the generation of antibodies capable of blocking virus entry/infection of the target cells. These antibodies blocking virus entry are known as neutralizing antibodies. The effector components of cell-mediated immunity, CD4 and CD8 T cells, mediate their antiviral effect through the secretion of cytokines and the killing of virus-infected target cells.

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56003-5

There is a fundamental difference in the antiviral response between humoral and cell-mediated immunity. Neutralizing antibodies are critical in preventing virus infection and have poor/no effect in controlling established chronic infection while virus-specific CD4 and CD8 T cells do not prevent infection but are critical in the control of chronic infection.

The most diffuse human viruses have indeed different susceptibilities to the effector components of humoral and cell-mediated immunity based on their ability to persist and establish chronic infection. Examples of viruses that are rapidly cleared by the immune response during primary infection include influenza virus, poliovirus, measles virus, rubella virus, poxvirus, hepatitis A virus, and yellow fever virus (Arvin, 1996; Griffin, 1995; Pantaleo and Koup, 2004). HIV-1 belongs to the class of viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), hepatitis B (HBV) and C (HCV) viruses, varicella zoster virus (VSV), and human papilloma virus (HPV) that persist and establish chronic infection (Bertoletti and Ferrari, 2003; Callan, 2003; Doherty *et al.*, 2001; Harari *et al.*, 2004b; Pantaleo and Koup, 2004). The ability of certain viruses to establish chronic infection results from a variety of mechanisms that viruses have evolved to evade the host immune response (Alcami and Koszinowski, 2000; Bachmann and Kopf, 1999; Bachmann and Zinkernagel, 1997; Doherty and Christensen, 2000; McMichael and Phillips, 1997; Walker and Goulder, 2000; Zinkernagel *et al.*, 2001). The virus immune escape mechanisms operate at two levels allowing both the persistence of the virus at the time of the first virus/host encounter and the inefficient control of virus replication during the established chronic infection.

Interestingly, there is a substantial difference in the immune response to viruses that are efficiently cleared during primary and chronic infection (Pantaleo and Koup, 2004). The immune response against viruses that are efficiently cleared at the time of primary infection is predominantly mediated by neutralizing antibody (Pantaleo and Koup, 2004). Neutralizing antibodies are also primarily responsible for protection from infection following vaccination. The effectiveness of neutralizing antibodies is related to the biology of the virus and to the type of infection. For instance, viruses that are rapidly cleared during primary infection generally are highly cytopathic and induce massive death of the target cells and release virus particles that in turn become susceptible to the neutralizing activity of antibodies. These viruses are not capable of establishing chronic infection in the target cells and therefore cell-mediated immunity has limited effect against these viruses.

Viruses that establish chronic infection are capable of establishing chronic infection in the target cells and cell-mediated immunity is the optimal response against cell-associated viruses. The protective effects of neutralizing antibodies against cell-associated viruses remain unclear.

The present chapter focuses on the analysis of HIV-1-specific immune response. In particular, we will analyze both HIV-1-specific humoral and cell-mediated immunity. With regard to the HIV-1-specific T-cell response,

we will review the current strategies, both phenotypic and functional, to analyze virus-specific T-cell responses, the phenotypic and functional abnormalities observed in the HIV-1-specific T-cell responses compared with other chronic infections that are efficiently controlled by the immune response, and finally, the immune correlates of protective virus-specific T-cell immune responses.

II. Humoral HIV-1-Specific Response

The immune correlates of protection from infection of the effective vaccines that have been developed against a variety of viruses are represented by neutralizing antibodies (Letvin, 2006; McMichael, 2006; Pantophlet and Burton, 2006). Neutralizing antibodies either prevent infection or may effectively contain the spreading of infection following virus exposure. They are predominantly effective against free virus particles while they have limited effects against virus-infected cells (Bachmann and Kopf, 1999; Bachmann and Zinkernagel, 1997; Zinkernagel *et al.*, 2001). The humoral immune response to HIV-1 is characterized by the presence of a vigorous antibody response against several viral proteins (Pantophlet and Burton, 2006). The presence of HIV-1 antibodies is indeed used as screening laboratory assay for the diagnosis of HIV-1 infection. However, the large majority of the antibody response is composed of antibodies lacking neutralizing activity.

The protective role of neutralizing antibodies in chronic HIV-1 infection is unclear. Neutralizing antibodies are generally not found in subjects with progressive infection while high titers of neutralizing antibodies with broad neutralizing activity are found in subjects with nonprogressive HIV-1 infection, that is, long-term nonprogressors (LTNPs) (Carotenuto *et al.*, 1998).

Several studies have investigated the mechanisms responsible for the poor neutralizing response and for the narrowed neutralizing activity against different HIV-1 primary isolates. This is particularly relevant for vaccine development. In this regard, protein-based candidate vaccines have been used to induce antibody responses (Letvin, 2006; McMichael, 2006; Pantophlet and Burton, 2006). However, the results obtained with the individual vaccines that have already been tested in phase III efficacy clinical trials have been disappointing in terms of the magnitude and of the neutralizing activity of the antibody responses elicited (Letvin, 2005). Therefore, there are three major problems with the antibody responses induced by the current protein-based vaccines: (1) poor magnitude, (2) poor/absent neutralizing activity, and (3) lack of broad neutralizing activity. In this regard, it is important to mention that neutralizing activity in the serum of HIV-1-infected humans is not detected until the ELISA titer is $>$1:100,000. Two major mechanisms have been proposed to explain HIV-1 antibody evasion: (1) the presence of V1-V3 variable regions and (2) the glycan shield. With regard to the first mechanism, it has

been proposed that the coreceptor binding site is masked by the V1-V2 variable regions. With regard to the glycan shield, 50% of the gp120 is covered by carbohydrates that render the surface of gp120 inaccessible to antibodies.

Therefore, eliciting robust neutralizing antibody responses against HIV in particular remains a major stumbling block for vaccine development (Burton *et al.*, 2004). Only a few human monoclonal antibodies have shown broad enough potency. These neutralizing antibodies have targeted conserved regions on the HIV envelope glycoproteins. The neutralizing antibodies IgG1b12 (Burton *et al.*, 1994) and 2G12 (Trkola *et al.*, 1996) target gp120, whereas 2F5, 4E10, and Z13 (and Z13i) target a membrane-proximal external region (MPER) of gp41 (Buchacher *et al.*, 1994; Muster *et al.*, 1993; Purtscher *et al.*, 1994; Stiegler *et al.*, 2001; Zwick *et al.*, 2001). A series of observations have clearly shown that these antibodies are protective. First, it has been shown that passive transfer of neutralizing antibodies can completely protect against lentiviral challenge in nonhuman primates (Zwick *et al.*, 2005). Second, an important finding in a recent clinical study has shown that administration of a high dose cocktail of 2F5, 4E10, and 2G12 could significantly delay rebound after cessation of antiviral therapy demonstrating protective potential of the humoral response (Trkola *et al.*, 2005). This finding demonstrates that the target epitopes are indeed present on the circulating virus envelopes and suggests that one might be able to induce neutralizing antibodies to these and other epitopes if appropriate strategies of immunization were employed.

III. HIV-1-Specific T-Cell Responses

A. Protective Role of HIV-1-Specific T-Cell Responses

A large number of studies strongly support the protective role of HIV-1-specific T-cell responses in the control of virus replication and in the prevention of HIV-1-associated disease (McMichael *et al.*, 2000; Migueles *et al.*, 2002; Pantaleo and Harari, 2006; Pantaleo and Koup, 2004; Rosenberg *et al.*, 1997, 2000). Certain functional profiles of HIV-1-specific CD4 and CD8 T-cell responses have been shown to correlate with more effective control of virus replication and stable disease (Appay *et al.*, 2000; Betts *et al.*, 2006; Harari *et al.*, 2004c, 2005, 2006; Iyasere *et al.*, 2003; Lichterfeld *et al.*, 2004; Migueles *et al.*, 2002; Pantaleo and Harari, 2006; Pantaleo and Koup, 2004; Rosenberg *et al.*, 1997; Younes *et al.*, 2003; Zimmerli *et al.*, 2005). With regard to CD4 T cells, vigorous HIV-1-specific CD4 T-cell proliferative responses correlate with lower levels of viral load and more effective control of virus replication following primary infection (Rosenberg *et al.*, 1997). Furthermore, it has also been shown that a critical component of protective HIV-1-specific CD4 T-cell responses is represented

by the presence of IL-2 secreting CD4 T cells (see the later description). Vigorous HIV-1-specific CD4 T-cell responses are also consistently found in LTNPs (Harari *et al.*, 2004c, 2005; Iyasere *et al.*, 2003; Tilton *et al.*, 2007).

The evidences for a protective role of HIV-1-specific T-cell responses are even stronger for CD8 T cells (Betts *et al.*, 1999; Pantaleo and Harari, 2006; Pantaleo and Koup, 2004). In particular, there are several observations supporting the protective role of HIV-1-specific CD8 T-cell responses: (1) vigorous HIV-1-specific CD8 T-cell responses composed of cytotoxic, proliferating, and IL-2 secreting cells (see the later description) are found in LTNPs (Zimmerli *et al.*, 2005); (2) HIV-specific CD8 T-cell responses are found in subjects repetitively exposed to HIV-1 but remaining uninfected (Kaul *et al.*, 2004; Makedonas *et al.*, 2005); (3) the temporal association between the appearance of the HIV-1-specific CD8 T-cell response and suppression of viral load during primary infection (McMichael *et al.*, 2000); (4) the rapid progression of disease following the emergence of virus escape mutants to the CD8 T-cell response (McMichael *et al.*, 2000); and (5) depletion of CD8 T cells in monkeys infected with simian immunodeficiency virus is associated with loss of control of virus replication and high levels of viral load (Schmitz *et al.*, 2005).

B. Kinetics of HIV-1-Specific T-Cell Responses in Primary Infection

Vigorous HIV-1-specific T-cell responses and particularly CD8 T-cell responses are associated with primary infection (Gandhi and Walker, 2002). In this regard, it has been shown that HIV-1-specific CD8 T cells can be massively expanded and represented up to 40% of total CD8 T cells. As mentioned earlier, the peak of CD8 T cell response is correlated with the decline of the high levels of viremia, and it has certainly an important role in the initial suppression of virus replication (Pantaleo *et al.*, 1994). The response of the massively expanded HIV-1-specific CD8 T-cell population during primary infection is mostly directed against a single epitope (Cao *et al.*, 2003). The initially expanded HIV-1-specific CD8 T-cell population progressively reduces as viremia levels decline. Therefore, it is clear that this initial CD8 T-cell response is very powerful, and it is likely that this response exerts high selective pressure as indicated by viral sequence diversification and eventually emergence of virus escape mutants. After the transition to the chronic phase of infection, the magnitude of the HIV-1-specific CD8 T-cell response is generally lower compared to primary infection. However, substantial changes occur in the specificity and breadth of the CD8 T-cell response (Addo *et al.*, 2003; Cao *et al.*, 2003). HIV-1-specific CD8 T-cell responses are directed against different HIV-1 proteins and against multiple epitopes. Therefore, there is broader specificity and larger breadth of the HIV-specific CD8 T-cell response during chronic infection (Altfeld *et al.*, 2001). Emergence of virus escape mutants occurs less frequently during chronic infection thus indicating the presence of

limited selective virus pressure mediated by the HIV-1-specific CD8 T-cell response. In addition, these observations are in favor of a less efficient CD8 T-cell response. In this regard, a recent study has shown a shift from high-avidity to low-avidity HIV-1-specific CD8 T cells from the primary to the chronic phase of infection consistently with a lower efficiency of the CD8 T-cell response during chronic infection (Lichterfeld *et al.*, 2007).

The kinetics of the HIV-1-specific CD4 T-cell response are certainly heavily influenced by the initial massive depletion of CD4 T cells associated with primary infection (Brenchley *et al.*, 2004; Mattapallil *et al.*, 2005). As mentioned earlier, the presence of HIV-1-specific proliferating CD4 T cells is associated with better control of the primary infection (Rosenberg *et al.*, 2000). In addition, these CD4 T cells also secrete IFN-γ (see the later description) (Pitcher *et al.*, 1999). With regard to the specificity of the response, it is predominantly directed against gag while it has been shown that env-specific CD4 T-cells responses rapidly disappear (Kaufmann *et al.*, 2004).

C. Phenotypic and Functional Profiles of HIV-1-Specific CD4 and CD8 T-Cell Responses

The characterization of the HIV-1-specific T-cell responses has been the objective of a very large number of studies and any type of phenotypic and functional abnormalities have been described (Appay *et al.*, 2002; Autran *et al.*, 1995, Brenchley *et al.*, 2003; Champagne *et al.*, 2001; Gillespie *et al.*, 2000; Hamann *et al.*, 1997; Harari *et al.*, 2004a, 2006; van Baarle *et al.*, 2002; van Lier *et al.*, 2003; Weekes *et al.*, 1999a,b; Zimmerli *et al.*, 2005). For these reasons, it is important to provide some general background on the phenotypic markers and functions that are generally used to define populations of antigen (Ag)-specific T cells at different stages of differentiation prior to address in details the abnormalities observed in HIV infection.

D. Phenotype

The term "memory T cells" is generally used to define antigen-experienced T cells that are present after the contraction of the immune response that is associated with antigen elimination or effective clearance (Sprent and Surh, 2002; Zinkernagel *et al.*, 1996). Both these conditions reflect either pathogen elimination or immune-mediated effective control. Therefore, caution should be used using the term of "memory T cells" in HIV infection where the pathogen is neither eliminated nor effectively controlled. For these reasons, we think that the term "antigen-specific" and not memory T cell is more appropriate to discuss the phenotypic profile of HIV-1-specific T cells.

The markers that have been extensively used to define antigen-specific CD4 and CD8 T cells include CD45RO, CD45RA, CD62L, CD28, CD27, CD7, CD57, CD127, and CCR7 (Appay *et al.*, 2002; Autran *et al.*, 1995;

Brenchley *et al.*, 2003; Champagne *et al.*, 2001; Gillespie *et al.*, 2000; Hamann *et al.*, 1997; Harari *et al.*, 2004a, 2006; van Baarle *et al.*, 2002; van Lier *et al.*, 2003; Weekes *et al.*, 1999a,b; Zimmerli *et al.*, 2005). The use of these markers has been instrumental to identify antigen-specific T cells at different stages of differentiation. As shown in Fig. 1, four levels of T-cell differentiation—early, intermediate, advanced, and terminal—have been defined and the general rule is that the above listed T-cell markers are progressively lost during differentiation (Kaech *et al.*, 2002; Klenerman and Hill, 2005; van Lier *et al.*, 2003). CD4 and CD8 T cells at early stage of differentiation express CD45RA/RO, CD28, CD27, CD7, CD127, and CCR7; at intermediate stage CD45RO, CCR7, and CD27; at advanced stage CD45RO; and at terminal stage acquire CD45RA and CD57 (Harari

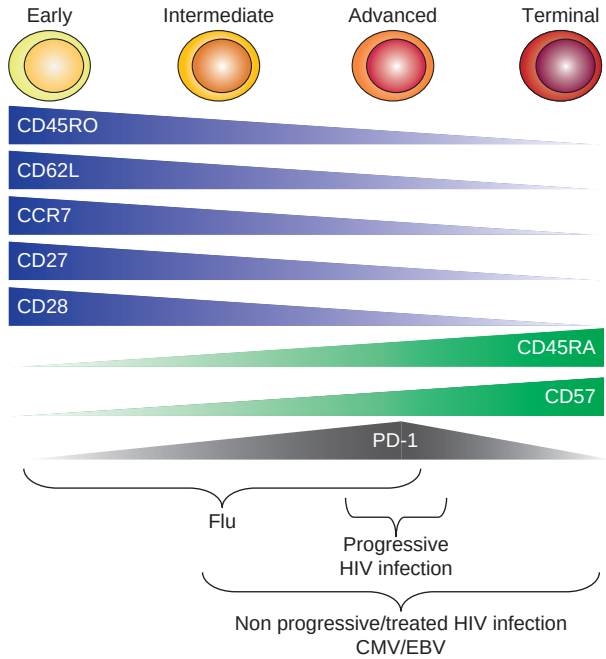


FIGURE 1 Relationship between phenotypic markers, stages of differentiation, and phenotype of virus-specific CD4/8 T cells. Early stage differentiation is associated with the expression of the majority of markers. Differentiation is associated with the progressive loss of a large number of markers. Expression of CD45RA and CD57 in the absence of the other markers defines a terminal differentiation stage.

et al., 2006; Kaech *et al.*, 2002; Klenerman and Hill, 2005; van Lier *et al.*, 2003). The CD57 marker has been associated with T-cells senescence (Brenchley *et al.*, 2003).

The combined use of CCR7 and CD45RA has been the first combination of markers leading to the identification of two phenotypically and functionally distinct populations of antigen-specific T cells (Sallusto *et al.*, 2004, 1999). The distinction was prevalently based upon the expression of CCR7. CD4 and CD8 T-cell populations expressing CCR7 are termed central memory (T_{CM}) and T cells lacking CCR7 are termed effector memory (T_{EM}) (Sallusto *et al.*, 2004, 1999). It is important to underscore that T_{CM} reside predominantly in the secondary lymphoid organs and are at earlier stage of differentiation compared with T_{EM} that concentrate in the periphery and at the site of infection. CD27 has been used in combination with CD45RA/RO to define populations of T_{CM} and T_{EM} (van Baarle *et al.*, 2002; van Lier *et al.*, 2003). However, CD27 and CCR7 do not identify similar populations of T_{CM} and T_{EM} and therefore caution should be used in comparing the results of studies using these two markers (Harari *et al.*, 2006).

CD127, the IL-7Rα, is a valuable marker and it has been shown to be expressed on long-lived memory CD4 and CD8 T cells (Kaech *et al.*, 2003).

The combined use of CD45RA/RO, CCR7, and CD127 defines four distinct populations of antigen-specific CD4 and CD8 T cells: (1) T_{CM} defined by the CD45RA−/RO+CD127+CCR7+ phenotype, (2) T_{EM} defined by the CD45RA−/RO+CD127−/+CCR7−phenotype, (3) effector terminal (T_{ET}) defined by the CD45RA+/RO−CD127−CCR7− phenotype, and (4) effectors (E) defined by the CD45RA−/RO+CD127−CCR7−phenotype.

The phenotype of HIV-1-specific CD4 T cells during primary infection is typical of effector cells and thus CD45RA−CCR7−CD127− (Harari *et al.*, 2004a, 2005, 2006). This phenotypic profile is not different from that observed for CMV-specific CD4 T cells during CMV primary infection (Harari *et al.*, 2004a, 2005, 2006). Therefore, the phenotype of HIV-1-specific CD4 T cell does not have any abnormality, and it is consistent with a typical effector primary T-cell response during the first pathogen encounter (Fig. 1).

However, in chronic infection, the phenotype of HIV-1-specific CD4 T cells is substantially different from that observed for virus-specific CD4 T cells against CMV, EBV, and HSV that although establish chronic infection are efficiently controlled by the immune response (Appay *et al.*, 2002; Klenerman and Hill, 2005; van Lier *et al.*, 2003). The phenotype of HIV-1-specific CD4 T cells remain unchanged in chronic infection compared with primary infection (Harari *et al.*, 2004a, 2006). Therefore, it is a typical phenotype of effector cells, and this is consistent with both the lack of elimination and of control of HIV-1 during chronic infection. The more effective control of virus replication in the case of chronic HIV-1 infection in LTNPs and of HSV infection is reflected by virus-specific CD4 T cells with

both T_{CM} and T_{EM} phenotypes (Harari *et al.*, 2004a, 2006). In support of a more effective control of virus replication during chronic infection, the phenotype of CMV- and EBV-specific CD4 T cells is consistent with that of T_{CM}, T_{EM}, and T_{ET} CD4 T cells (Harari *et al.*, 2004a, 2006).

Similar considerations can be made for the phenotypic profile of HIV-specific CD8 T cells during primary and chronic infection. The phenotype of HIV-1- and CMV-specific CD8 T cells during primary infection is typical of effector cells and thus CD45RA–CCR7–CD127– and thus consistent with a typical primary T-cell response. As for CD4 T cells, the phenotype of HIV-1-specific CD8 T cells remain unchanged in chronic infection compared with primary infection consistently with the persistence of HIV-1 and with the lack of control of virus replication (Zimmerli *et al.*, 2005). However, HIV-1-specific CD8 T cells from LTNPs and influenza-specific CD8 T cells have phenotypic profiles characteristic of T_{CM} and T_{EM}. Finally, the phenotype of CMV- and EBV-specific CD8 T cells is consistent with that of T_{CM}, T_{EM}, and T_{ET} CD8 T cells (Harari *et al.*, 2006).

Therefore, there is a good correlation between the phenotypic profiles of both virus-specific CD4 and CD8 T cells and the control of virus replication. In this regard, it is worth to mention that a number of studies have shown a correlation between the magnitude of the HIV-1-specific T_{ET} CD8 T-cell population and virus control, that is, low viremia levels, in subjects with chronic infection (Champagne *et al.*, 2001; Ellefsen *et al.*, 2002).

E. Function

Over the past years, a series of functions have been shown to be relevant in antiviral immunity (Appay *et al.*, 2000; Betts *et al.*, 2003, 2004; Harari *et al.*, 2004c, 2005, 2006; Iyasere *et al.*, 2003; Lichterfeld *et al.*, 2004; Mallard *et al.*, 2004; Migueles *et al.*, 2002; Palmer *et al.*, 2004; Pantaleo and Harari, 2006; Pantaleo and Koup, 2004; Seder and Ahmed, 2003; Suni *et al.*, 2005; Younes *et al.*, 2003; Zimmerli *et al.*, 2005). They include: (1) proliferation capacity, (2) cytokines/chemokines secretion including IL-2, IFN-γ, TNF-α, and MIP-1β, and (3) cytotoxicity as measured by perforin/granzyme B expression and CD107a/b mobilization/degranulation activity (Fig. 2).

On the basis of the analysis of IL-2 and IFN-γ, three functionally distinct populations of antigen-specific CD4 T cells (single IL-2, dual IL-2/IFN-γ, and single IFN-γ) have been identified (Betts *et al.*, 2006; Harari *et al.*, 2004c, 2005, 2006; Pantaleo and Harari, 2006; Younes *et al.*, 2003; Zimmerli *et al.*, 2005). Furthermore, the presence of IL-2 secreting T cells is consistently associated with the antigen-specific proliferation capacity (Harari *et al.*, 2004c, 2006; Zimmerli *et al.*, 2005). Single IL-2 and dual IL-2/IFN-γ antigen-specific CD4 T-cell populations have intrinsic proliferation capacity while single IFN-γ have poor proliferation capacity that can be promoted in the presence of an exogenous source of IL-2 (Harari *et al.*, 2004c, 2006; Zimmerli *et al.*, 2005).

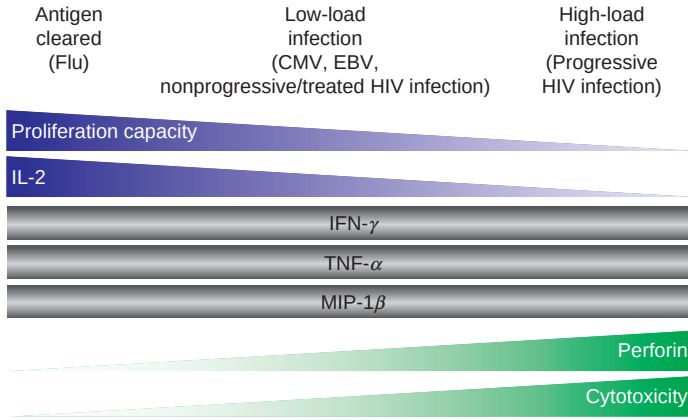


FIGURE 2 Schematic representation of the functional profile of virus-specific CD4 and CD8 T cells based on the level/duration of antigen exposure/load. All functions are relevant for both CD4 and CD8 T cells with the exception of perforin expression and cytotoxicity which pertain to CD8 T cells.

A large percentage (>60%) of antigen-specific CD4 T cells secrete TNF-α and, based on the secretion of IFN-γ, two equally represented cell populations of single IFN-γ and dual IFN-γ/TNF-α can be identified.

With regard to CD8 T cells, two cell populations of antigen-specific CD8 T cells (dual IL-2/IFN-γ and single IFN-γ) can be defined based on the ability to secrete IL-2 and IFN-γ (Zimmerli *et al.*, 2005). Both CD8 T-cell populations are cytotoxic as measured by the expression of perforin and granzyme B or by the degranulation activity following antigen-specific stimulation (Harari *et al.*, 2006). However, the expression of these markers of cytotoxic function is greater in the single IFN-γ compared with dual IL-2/IFN-γ CD8 T cells. The dual IL-2/IFN-γ CD8 T cells proliferate following antigen-specific stimulation through an autocrine mechanism of IL-2 secretion and their proliferation is CD4 independent (Harari *et al.*, 2006; Pantaleo and Harari, 2006; Zimmerli *et al.*, 2005). Single IFN-γ CD8 T cells have no intrinsic proliferation capacity, and their proliferation can be rescued in the presence of antigen-specific CD4 T cells and exogenous IL-2 (Harari *et al.*, 2006; Pantaleo and Harari, 2006; Zimmerli *et al.*, 2005). Furthermore, virtually the totality of TNF-α secreting cells were also IFN-γ secreting cells, that is, dual IFN-γ/TNF-α. Finally, the assessment of MIP-1β has been proposed to define a variable percentage (up to 30%) of antigen-specific CD8 T cells not producing IL-2, TNF-α, and IFN-γ (Betts *et al.*, 2006).

Recently, the term polyfunctional has been used to define CD4 and CD8 T-cell responses that, in addition to typical effector functions such as secretion of IFN-γ, TNF-α, MIP-1β, and cytotoxic activity, comprise distinct

T-cell populations also able to secrete IL-2 and retaining antigen-specific proliferation capacity. The term "only effector" defines T-cell responses/populations able to secrete cytokines such as IFN-γ, TNF-α, and MIP-1β, and endowed with cytotoxic activity but lacking IL-2 and proliferation capacity.

The patterns of HIV-1-specific CD4 T-cell response during primary infection are typical of an "only effector" response, that is, IFN-γ and TNF-α secreting CD4 T cells lacking proliferation capacity. This pattern is not different from that observed for the primary CMV-specific CD4 T-cell response. The patterns of HIV-1-specific CD4 T-cell response during chronic infection are dependent upon the degree of control of virus replication. In the absence of control of virus replication, the HIV-1-specific CD4 T-cell response remains unchanged compared with primary infection and maintains an effector profile. In the presence of effective control of virus replication following antiviral therapy or in LTNPs, the profile of the HIV-1-specific CD4 T-cell response becomes polyfunctional (Harari *et al.*, 2005, 2006; Tilton *et al.*, 2007). Suppression of virus replication is therefore associated with the presence of single IL-2 and dual IL-2/IFN-γ secreting HIV-1-specific CD4 T-cell populations and of cells endowed with proliferation capacity (Harari *et al.*, 2005, 2006; Tilton *et al.*, 2007). These observations strongly support the hypothesis that virus replication and high levels of viral load prevent the generation of IL-2 secreting and proliferating HIV-1-specific CD4 T cells (Harari *et al.*, 2005, 2006; Tilton *et al.*, 2007). The development of a polyfunctional pattern of HIV-1-specific CD4 T-cell response only under conditions of effective suppression of virus replication is also consistent with the patterns of virus-specific CD4 T-cell responses observed in a series of chronic virus infections such as CMV, EBV, HSV, and HCV (Harari *et al.*, 2005, 2006; Tilton *et al.*, 2007). In all instances in these models of chronic virus infections, the presence of polyfunctional CD4 T-cell responses is associated with more effective control of virus replication (Harari *et al.*, 2005, 2006; Tilton *et al.*, 2007) (Fig. 3).

Similar to CD4 T-cell responses, the patterns of HIV-1-specific CD8 T-cell response during primary infection are typical of an "only effector" response, that is, IFN-γ and TNF-α secreting CD8 T cells and not different from that observed for the primary CMV-specific CD8 T-cell response. The patterns of HIV-1-specific CD8 T-cell response during the chronic phase of HIV-1 infection were only in part related to the extent of viral load (Pantaleo and Harari, 2006). As for CD4 T-cell response, the patterns of HIV-1-specific CD8 T-cell response remain typical of an effector response in the absence of control of virus replication and high viral load (Zimmerli *et al.*, 2005). However, development of polyfunctional HIV-1-specific CD8 T-cell responses occurs only in 30–40% of subjects that are successfully treated with antiviral therapy (Pantaleo and Harari, 2006). Therefore, polyfunctional HIV-1-specific CD8 T-cell responses are not recovered in a large

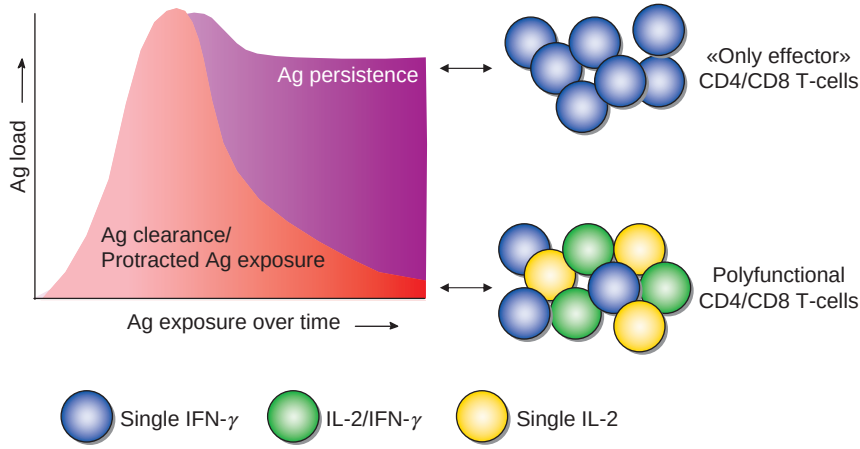


FIGURE 3 Association between the level of antigen exposure and the functional profile of virus-specific CD4 and CD8 T cells. Single IFN-γ and dual IFN-γ/IL-2 are relevant for both CD4 and CD8 T cells while single IL-2 secreting T cells are only considered for CD4 T cells.

percentage of subjects despite suppression of virus replication and of viral load (Zimmerli *et al.*, 2005). These findings are different from those obtained for HIV-1-specific CD4 T-cell responses that were consistently associated with the recovery of polyfunctional responses after antiviral therapy-mediated suppression of virus replication. Polyfunctional HIV-1-specific CD8 T-cell responses are, however, consistently found in LTNPs (Betts *et al.*, 2006; Zimmerli *et al.*, 2005). Furthermore, virus-specific polyfunctional CD8 T-cell responses are also found in virus infections such as influenza, CMV, EBV in which the virus is either completely eliminated or efficiently controlled after establishment of chronic infection (Harari *et al.*, 2006; Pantaleo and Harari, 2006).

Taken together these observations indicate that, as for CD4 T cells, polyfunctional CD8 T-cell responses are also consistently associated with more effective control of virus replication (Fig. 3). However, in addition to viral load, other factors seem to influence the development of polyfunctional CD8 T-cell responses.

F. Specificity and Breadth of HIV-1-Specific T-Cell Responses

Extensive characterization of the specificity and the breadth of HIV-1-specific T-cell responses have been performed particularly for CD8 T cells. HIV-1-specific CD8 T cells recognize a large number of epitopes within the different HIV-1 proteins including structural, regulatory, and accessory proteins. In this regard, a recent study has shown an association between

the presence of gag-specific CD8 T-cell responses and lower levels of viremia (Kiepiela *et al.*, 2007). In contrast, CD8 T-cell responses against env and accessory/regulatory proteins were associated with higher viremia levels (Kiepiela *et al.*, 2007). These observations are of interest and indicate the possibility that immune responses targeting certain regions of HIV-1 may be more protective than others and eventually influence the clinical course of chronic HIV-1 infection. However, it is unclear whether the gag-specific CD8 T-cell responses are directly responsible for the better control of virus replication or whether the env-specific CD8 T-cell responses are truly less effective. It cannot be excluded that the appearance of the env-specific responses is rather the result and not the cause of higher virus replication. In support of previous studies, it has also been shown that at least for gag-specific CD8 T-cell responses, a larger breadth of the CD8 T-cell response is associated with more effective control of virus replication.

References

Addo, M. M., Yu, X. G., Rathod, A., Cohen, D., Eldridge, R. L., Strick, D., Johnston, M. N., Corcoran, C., Wurcel, A. G., Fitzpatrick, C. A., Feeney, M. E., Rodriguez, W. R., *et al.* (2003). Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J. Virol.* **77**, 2081–2092.

Alcami, A., and Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. *Immunol. Today* **21**, 447–455.

Altfeld, M., Rosenberg, E. S., Shankarappa, R., Mukherjee, J. S., Hecht, F. M., Eldridge, R. L., Addo, M. M., Poon, S. H., Phillips, M. N., Robbins, G. K., Sax, P. E., Boswell, S., *et al.* (2001). Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J. Exp. Med.* **193**, 169–180.

Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., Spiegel, H. M., Conlon, C., Spina, C. A., Havlir, D. V., Richman, D. D., *et al.* (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* **192**, 63–75.

Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A., Lechner, F., Spina, C. A., Little, S., Havlir, D. V., *et al.* (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* **8**, 379–385.

Arvin, A. M. (1996). Immune responses to varicella-zoster virus. *Infect. Dis. Clin. North Am.* **10**, 529–570.

Autran, B., Legac, E., Blanc, C., and Debre, P. (1995). A Th0/Th2-like function of CD4+CD7− T helper cells from normal donors and HIV-infected patients. *J. Immunol.* **154**, 1408–1417.

Bachmann, M. F., and Kopf, M. (1999). The role of B cells in acute and chronic infections. *Curr. Opin. Immunol.* **11**, 332–339.

Bachmann, M. F., and Zinkernagel, R. M. (1997). Neutralizing antiviral B cell responses. *Annu. Rev. Immunol.* **15**, 235–270.

Bertoletti, A., and Ferrari, C. (2003). Kinetics of the immune response during HBV and HCV infection. *Hepatology* **38**, 4–13.

Betts, M. R., Krowka, J. F., Kepler, T. B., Davidian, M., Christopherson, C., Kwok, S., Louie, L., Eron, J., Sheppard, H., and Frelinger, J. A. (1999). Human immunodeficiency virus type

1-specific cytotoxic T lymphocyte activity is inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS Res. Hum. Retroviruses* **15**, 1219–1228.

Betts, M. R., Brenchley, J. M., Price, D. A., De Rosa, S. C., Douek, D. C., Roederer, M., and Koup, R. A. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* **281**, 65–78.

Betts, M. R., Price, D. A., Brenchley, J. M., Lore, K., Guenaga, F. J., Smed-Sorensen, A., Ambrozak, D. R., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C., Koup, R. A., *et al.* (2004). The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. *J. Immunol.* **172**, 6407–6417.

Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M., Koup, R. A., *et al.* (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781–4789.

Brenchley, J. M., Karandikar, N. J., Betts, M. R., Ambrozak, D. R., Hill, B. J., Crotty, L. E., Casazza, J. P., Kuruppu, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C., *et al.* (2003). Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* **101**, 2711–2720.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T., and Douek, D. C. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* **200**, 749–759.

Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., Jungbauer, A., *et al.* (1994). Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res. Hum. Retroviruses* **10**, 359–369.

Burton, D. R., Pyati, J., Koduri, R., Sharp, S. J., Thornton, G. B., Parren, P. W., Sawyer, L. S., Hendry, R. M., Dunlop, N., Nara, P. L., *et al.* (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* **266**, 1024–1027.

Burton, D. R., Desrosiers, R. C., Doms, R. W., Koff, W. C., Kwong, P. D., Moore, J. P., Nabel, G. J., Sodroski, J., Wilson, I. A., and Wyatt, R. T. (2004). HIV vaccine design and the neutralizing antibody problem. *Nat. Immunol.* **5**, 233–236.

Callan, M. F. (2003). The evolution of antigen-specific CD8+ T cell responses after natural primary infection of humans with Epstein-Barr virus. *Viral Immunol.* **16**, 3–16.

Cao, J., McNevin, J., Holte, S., Fink, L., Corey, L., and McElrath, M. J. (2003). Comprehensive analysis of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-secreting CD8+ T cells in primary HIV-1 infection. *J. Virol.* **77**, 6867–6878.

Carotenuto, P., Looij, D., Keldermans, L., de Wolf, F., and Goudsmit, J. (1998). Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection. *AIDS* **12**, 1591–1600.

Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., *et al.* (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* **410**, 106–111.

Doherty, P. C., and Christensen, J. P. (2000). Accessing complexity: The dynamics of virus-specific T cell responses. *Annu. Rev. Immunol.* **18**, 561–592.

Doherty, P. C., Christensen, J. P., Belz, G. T., Stevenson, P. G., and Sangster, M. Y. (2001). Dissecting the host response to a gamma-herpesvirus. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**, 581–593.

Ellefsen, K., Harari, A., Champagne, P., Bart, P. A., Sekaly, R. P., and Pantaleo, G. (2002). Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections. *Eur. J. Immunol.* **32**, 3756–3764.

Gandhi, R. T., and Walker, B. D. (2002). Immunologic control of HIV-1. *Annu. Rev. Med.* **53**, 149–172.

Gillespie, G. M., Wills, M. R., Appay, V., O'Callaghan, C., Murphy, M., Smith, N., Sissons, P., Rowland-Jones, S., Bell, J. I., and Moss, P. A. (2000). Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J. Virol.* **74**, 8140–8150.

Griffin, D. E. (1995). Immune responses during measles virus infection. *Curr. Top. Microbiol. Immunol.* **191**, 117–134.

Hamann, D., Baars, P. A., Rep, M. H., Hooibrink, B., Kerkhof-Garde, S. R., Klein, M. R., and van Lier, R. A. (1997). Phenotypic and functional separation of memory and effector human CD8+ T cells. *J. Exp. Med.* **186**, 1407–1418.

Harari, A., Vallelian, F., and Pantaleo, G. (2004a). Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur. J. Immunol.* **34**, 3525–3533.

Harari, A., Zimmerli, S. C., and Pantaleo, G. (2004b). Cytomegalovirus (CMV)-specific cellular immune responses. *Hum. Immunol.* **65**, 500–506.

Harari, A., Petitpierre, S., Vallelian, F., and Pantaleo, G. (2004c). Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: Changes after antiretroviral therapy. *Blood* **103**, 966–972.

Harari, A., Vallelian, F., Meylan, P. R., and Pantaleo, G. (2005). Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* **174**, 1037–1045.

Harari, A., Dutoit, V., Cellerai, C., Bart, P. A., Du Pasquier, R. A., and Pantaleo, G. (2006). Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* **211**, 236–254.

Iyasere, C., Tilton, J. C., Johnson, A. J., Younes, S., Yassine-Diab, B., Sekaly, R. P., Kwok, W. W., Migueles, S. A., Laborico, A. C., Shupert, W. L., Hallahan, C. W., Davey, R. T., Jr., *et al.* (2003). Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J. Virol.* **77**, 10900–10909.

Kaech, S. M., Wherry, E. J., and Ahmed, R. (2002). Effector and memory T-cell differentiation: Implications for vaccine development. *Nat. Rev. Immunol.* **2**, 251–262.

Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* **4**, 1191–1198.

Kaufmann, D. E., Bailey, P. M., Sidney, J., Wagner, B., Norris, P. J., Johnston, M. N., Cosimi, L. A., Addo, M. M., Lichterfeld, M., Altfeld, M., Frahm, N., Brander, C., *et al.* (2004). Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J. Virol.* **78**, 4463–4477.

Kaul, R., Rutherford, J., Rowland-Jones, S. L., Kimani, J., Onyango, J. I., Fowke, K., MacDonald, K., Bwayo, J. J., McMichael, A. J., and Plummer, F. A. (2004). HIV-1 Env-specific cytotoxic T-lymphocyte responses in exposed, uninfected Kenyan sex workers: A prospective analysis. *AIDS* **18**, 2087–2089.

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., Reddy, S., de Pierres, C., Mncube, Z., Mkhwanazi, N., Bishop, K., van der Stok, M., *et al.* (2007). CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13**, 46–53.

Klenerman, P., and Hill, A. (2005). T cells and viral persistence: Lessons from diverse infections. *Nat. Immunol.* **6**, 873–879.

Letvin, N. L. (2005). Progress toward an HIV vaccine. *Annu. Rev. Med.* **56**, 213–223.

Letvin, N. L. (2006). Progress and obstacles in the development of an AIDS vaccine. *Nat. Rev. Immunol.* **6**, 930–939.

Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., Cohen, D., Robbins, G. K., Pae, E., Alter, G., Wurcel, A., Stone, D., *et al.* (2004). Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *J. Exp. Med.* **200**, 701–712.

Lichterfeld, M., Yu, X. G., Mui, S. K., Williams, K. L., Trocha, A., Brockman, M. A., Allgaier, R. L., Waring, M. T., Koibuchi, T., Johnston, M. N., Cohen, D., Allen, T. M., *et al.* (2007). Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. *J. Virol.* **81**, 4199–4214.

Makedonas, G., Bruneau, J., Alary, M., Tsoukas, C. M., Lowndes, C. M., Lamothe, F., and Bernard, N. F. (2005). Comparison of HIV-specific CD8 T-cell responses among uninfected individuals exposed to HIV parenterally and mucosally. *AIDS* **19**, 251–259.

Mallard, E., Vernel-Pauillac, F., Velu, T., Lehmann, F., Abastado, J. P., Salcedo, M., and Bercovici, N. (2004). IL-2 production by virus- and tumor-specific human CD8 T cells is determined by their fine specificity. *J. Immunol.* **172**, 3963–3970.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., and Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093–1097.

McMichael, A. J. (2006). HIV vaccines. *Annu. Rev. Immunol.* **24**, 227–255.

McMichael, A. J., and Phillips, R. E. (1997). Escape of human immunodeficiency virus from immune control. *Annu. Rev. Immunol.* **15**, 271–296.

McMichael, A. J., Callan, M., Appay, V., Hanke, T., Ogg, G., and Rowland-Jones, S. (2000). The dynamics of the cellular immune response to HIV infection: Implications for vaccination. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 1007–1011.

Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., *et al.* (2002). HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol.* **3**, 1061–1068.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., and Katinger, H. (1993). A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J. Virol.* **67**, 6642–6647.

Palmer, B. E., Boritz, E., and Wilson, C. C. (2004). Effects of sustained HIV-1 plasma viremia on HIV-1 Gag-specific CD4+ T cell maturation and function. *J. Immunol.* **172**, 3337–3347.

Pantaleo, G., and Harari, A. (2006). Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat. Rev. Immunol.* **6**, 417–423.

Pantaleo, G., and Koup, R. A. (2004). Correlates of immune protection in HIV-1 infection: What we know, what we don't know, what we should know. *Nat. Med.* **10**, 806–810.

Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P., and Fauci, A. S. (1994). Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370**, 463–467.

Pantophlet, R., and Burton, D. R. (2006). GP120: Target for neutralizing HIV-1 antibodies. *Annu. Rev. Immunol.* **24**, 739–769.

Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C., and Picker, L. J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* **5**, 518–525.

Purtscher, M., Trkola, A., Gruber, G., Buchacher, A., Predl, R., Steindl, F., Tauer, C., Berger, R., Barrett, N., Jungbauer, A., *et al.* (1994). A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **10**, 1651–1658.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., and Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**, 1447–1450.

Rosenberg, E. S., Altfeld, M., Poon, S. H., Phillips, M. N., Wilkes, B. M., Eldridge, R. L., Robbins, G. K., D'Aquila, R. T., Goulder, P. J., and Walker, B. D. (2000). Immune control of HIV-1 after early treatment of acute infection. *Nature* **407**, 523–526.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–712.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: Function, generation, and maintenance. *Annu. Rev. Immunol.* **22**, 745–763.

Schmitz, J. E., Johnson, R. P., McClure, H. M., Manson, K. H., Wyand, M. S., Kuroda, M. J., Lifton, M. A., Khunkhun, R. S., McEvers, K. J., Gillis, J., Piatak, M., Lifson, J. D., *et al.* (2005). Effect of CD8+ lymphocyte depletion on virus containment after simian immunodeficiency virus SIVmac251 challenge of live attenuated SIVmac239delta3-vaccinated rhesus macaques. *J. Virol.* **79**, 8131–8141.

Seder, R. A., and Ahmed, R. (2003). Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat. Immunol.* **4**, 835–842.

Sprent, J., and Surh, C. D. (2002). T cell memory. *Annu. Rev. Immunol.* **20**, 551–579.

Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F., and Katinger, H. (2001). A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **17**, 1757–1765.

Suni, M. A., Maino, V. C., and Maecker, H. T. (2005). Ex vivo analysis of T-cell function. *Curr. Opin. Immunol.* **17**, 434–440.

Tilton, J. C., Luskin, M. R., Johnson, A. J., Manion, M., Hallahan, C. W., Metcalf, J. A., McLaughlin, M., Davey, R. T., Jr., and Connors, M. (2007). Changes in paracrine interleukin-2 requirement, CCR7 expression, frequency, and cytokine secretion of human immunodeficiency virus-specific CD4+ T cells are a consequence of antigen load. *J. Virol.* **81**, 2713–2725.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P., and Katinger, H. (1996). Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J. Virol.* **70**, 1100–1108.

Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., *et al.* (2005). Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat. Med.* **11**, 615–622.

van Baarle, D., Kostense, S., van Oers, M. H., Hamann, D., and Miedema, F. (2002). Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. *Trends Immunol.* **23**, 586–591.

van Lier, R. A., ten Berge, I. J., and Gamadia, L. E. (2003). Human CD8(+) T-cell differentiation in response to viruses. *Nat. Rev. Immunol.* **3**, 931–939.

Walker, B. D., and Goulder, P. J. (2000). AIDS. Escape from the immune system. *Nature* **407**, 313–314.

Weekes, M. P., Carmichael, A. J., Wills, M. R., Mynard, K., and Sissons, J. G. (1999a). Human CD28-CD8+ T cells contain greatly expanded functional virus-specific memory CTL clones. *J. Immunol.* **162**, 7569–7577.

Weekes, M. P., Wills, M. R., Mynard, K., Hicks, R., Sissons, J. G., and Carmichael, A. J. (1999b). Large clonal expansions of human virus-specific memory cytotoxic T lymphocytes within the CD57+ CD28− CD8+ T-cell population. *Immunology* **98**, 443–449.

Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P., and Sekaly, R. P. (2003). HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J. Exp. Med.* **198**, 1909–1922.

Zimmerli, S. C., Harari, A., Cellerai, C., Vallelian, F., Bart, P. A., and Pantaleo, G. (2005). HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc. Natl. Acad. Sci. USA* **102**, 7239–7244.

Zinkernagel, R. M., Bachmann, M. F., Kundig, T. M., Oehen, S., Pirchet, H., and Hengartner, H. (1996). On immunological memory. *Annu. Rev. Immunol.* **14**, 333–367.

Zinkernagel, R. M., LaMarre, A., Ciurea, A., Hunziker, L., Ochsenbein, A. F., McCoy, K. D., Fehr, T., Bachmann, M. F., Kalinke, U., and Hengartner, H. (2001). Neutralizing antiviral antibody responses. *Adv. Immunol.* **79**, 1–53.

Zwick, M. B., Bonnycastle, L. L., Menendez, A., Irving, M. B., Barbas, C. F., 3rd, Parren, P. W., Burton, D. R., and Scott, J. K. (2001). Identification and characterization of a peptide that specifically binds the human, broadly neutralizing anti-human immunodeficiency virus type 1 antibody b12. *J. Virol.* **75**, 6692–6699.

Zwick, M. B., Jensen, R., Church, S., Wang, M., Stiegler, G., Kunert, R., Katinger, H., and Burton, D. R. (2005). Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J. Virol.* **79**, 1252–1261.

Julie Strizki
Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Targeting HIV Attachment and Entry for Therapy

I. Chapter Overview

Over the past two decades, tremendous strides have been made in the development of potent antiviral drugs to treat HIV infection. Despite this progress, issues of toxicity, tolerability, and drug resistance have prompted researchers to continue the search for new and better ways to combat HIV infection and the growing global epidemic.

Recently, much attention has been focused on a new class of compounds known as entry inhibitors. These agents act at early steps in the viral life cycle to protect target cells from HIV infection. This chapter will provide background and rationale for targeting different stages of the HIV entry

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56004-7

process and provide an overview of the new agents that are being developed for the treatment and prevention of HIV infection.

II. Background

Inhibiting viral infection by interfering with the early steps in the viral life cycle has historically proved to be a valid strategy to fight viral diseases. Many viral vaccines are effective because they induce neutralizing antibodies that specifically bind to the viral surface proteins and prevent infection of target cells. One of the primary challenges for vaccine development is the propensity of viruses to mutate their surface proteins in order to evade neutralizing antibodies (Butler *et al.*, 2007; Moore *et al.*, 2001). Many viruses including HIV evade the immune system by continuously changing the exposed regions of the envelope protein or by masking functional epitopes with glycosylation (Frost *et al.*, 2005). Indeed, few antibodies with broad spectrum neutralizing activity against the HIV-1 envelope have been identified. Despite these challenges, scientific advances over the last decade have led to the discovery of multiple antiviral agents that target different points during the process of viral attachment and entry into target cells.

The envelope spike on the surface of the HIV particle consists of a trimer of heterodimers made up of the gp41 transmembrane glycoprotein that is noncovalently associated with the gp120 attachment protein. The molecular events involved in HIV infection have been intensively studied over the last decade, which has led to a better understanding of the process of viral attachment and membrane fusion (Markovic and Clouse, 2004). The viral entry process consists of three distinct stages including: (1) gp120 attachment to CD4, (2) coreceptor binding, and (3) gp41 rearrangement and fusion of the viral and cellular membranes (Doms and Moore, 2000; Fig. 1).

The first step in establishing a productive infection is attachment of the viral surface protein gp120 to the primary receptor, CD4, expressed on T cells or macrophages. This engagement of CD4 causes gp120 to undergo a stabilizing conformational change that exposes the binding sites for a secondary cellular coreceptor (Chen *et al.*, 2005). The primary coreceptors for HIV-1 are the chemokine receptors CXCR4 and CCR5 (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Following coreceptor binding, a second conformational change occurs which exposes the N-terminal fusion peptide in gp41, allowing it to then be inserted into the host cell membrane (Doms, 2001). The gp41 trimers then undergo another conformational change where the N-terminal and C-terminal heptad repeat regions come together to form a 6-helical coiled coil structure (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Formation of this helical bundle brings the viral and cellular membranes into proximity, facilitating

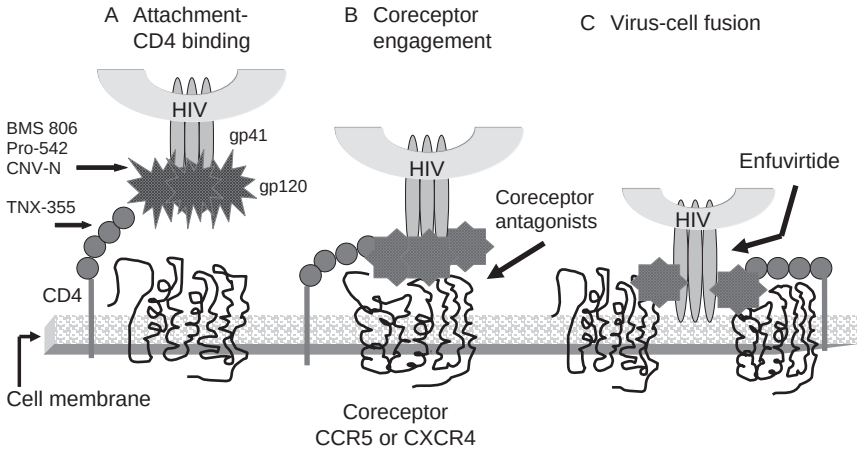


FIGURE 1 Targeting early steps in HIV infection. (A) HIV first attaches the cell via binding of the viral gp120 envelope protein to the CD4 receptor on the surface of target cells. Agents that target either gp120 (i.e., BMS 806, Pro-542, CNV-N) or antibodies against CD4 (TNX-355) can prevent the conformational changes in envelope required for coreceptor binding. (B) Following gp120-CD4 binding, a significant conformational change occurs in gp120 that exposes the residues important for engagement of the coreceptor (CCR5 or CXCR4). Small molecule antagonists, peptides, and antibodies that bind the coreceptor can inhibit this step of viral infection. (C) After gp120 binds to the coreceptor, a second conformational change occurs that allows for rearrangement of gp41 and insertion of the fusion peptide into the target cell membrane. The heptad repeat regions present in each of the gp41 monomers together to form a six-helical bundle that draws together the viral and cellular membranes and facilitates fusion pore formations. Inhibitors like enfuvirtide specifically bind to the heptad repeat regions in the prefusion conformation of g41 and prevent helical bundle formation and membrane fusion.

the fusion of the two membranes. Inhibitors to each of these steps have been identified and some have advanced to clinical trials.

III. Inhibition of Viral Attachment

The first specific interaction of HIV with the target cell involves binding of the gp120 envelope spike to the CD4 receptor on surface of the cell. Inhibition of this initial step in infection can be achieved by targeting either the gp120 protein or alternatively the CD4 receptor. Agents targeting both proteins have been identified and found to have antiviral activity.

A. gp120 Inhibitors

The gp120 envelope has proven to be a difficult protein to target for antiviral therapy for several reasons. First, gp120 is highly glycosylated (25–30 sites per molecule) with the sugar moieties forming a "glycan shield"

around the protein. This shield can mask antigenic epitopes and conserved functional sites on the protein (Wei *et al.*, 2003). In addition, gp120 sequences are genotypically heterogeneous, particularly in the variable loop regions that are more exposed on the outer surface of the protein (Huang *et al.*, 2005). These properties broadly make active agents against gp120 difficult to identify. Despite these hurdles, crystallographic studies of gp120 have revealed the presence of a relatively well-conserved CD4-binding pocket within the gp120 core (Rizzuto *et al.*, 1998). Residues within this pocket interact with the N-terminal domains of the CD4 receptor during viral attachment. One of the first strategies used to target gp120 took advantage of this conserved site by using a recombinant soluble form CD4 (sCD4) to neutralize viral particles (Clapham *et al.*, 1989; Deen *et al.*, 1988). This truncated sCD4 protein specifically binds to gp120, effectively coating the virion and preventing attachment to cells. Although sCD4 proved effective in neutralizing HIV *in vitro*, early clinical trials were disappointing, in part due to the short half-life of the molecule in the plasma (Kahn *et al.*, 1990; Schacker *et al.*, 1994; Schooley *et al.*, 1990). To remedy this issue, a group of researchers at Progenics engineered a chimeric molecule consisting of the extracellular domains of CD4 fused to the constant region of an immunoglobulin molecule (Allaway *et al.*, 1995). This hybrid, known as Pro-542, is a recombinant fusion protein generated by replacing the variable domains of an IgG2 molecule with the N-terminal D1 and D2 domains of CD4, thus forming a tetravalent CD4-IgG hybrid. This molecule demonstrates broad spectrum neutralization activity against HIV and is significantly more stable *in vivo* compared with sCD4 alone (Jacobson *et al.*, 2004). Pro-542 has entered clinical trials for treatment of heavily treatment-experienced patients and safety and long-term efficacy results are pending.

Another protein that inhibits HIV entry by interacting with gp120 is cyanovirin-N (Boyd *et al.*, 1997). This 11-kDa protein, isolated from cyanobacterium, binds to the sugar moieties on the gp120 surface and prevents gp120 from undergoing the conformational changes necessary for coreceptor binding and membrane fusion (Mori and Boyd, 2001; Shenoy *et al.*, 2001). However, cyanovirin-N is not specific for HIV gp120 and has been shown to inhibit infection of other enveloped viruses including, feline immunodeficiency virus and human herpes virus 6 (Dey *et al.*, 2000). Cyanovirin-N is currently being developed as a topical microbicide for prevention of HIV transmission (Liu *et al.*, 2006; Tsai *et al.*, 2004). Other peptidic inhibitors of viral entry that reportedly interact with gp120 are the theta defensins Retrocyclin-1 and RC-101. These are small cysteine-rich circular peptides that reportedly bind to glycosylated residues on gp120 (Cole *et al.*, 2007; Owen *et al.*, 2004; Wang *et al.*, 2003). Binding studies show that RC-101 binds with high affinity ($K_d \sim 30$–35 nM) to gp120 proteins from clade B viruses. However, RC-101 binding to nonclade B envelopes was substantially reduced ($K_d \sim 200$–700 nM). This difference

in binding affinity may be explained by differences in gp120 glycosylation patterns among the individual genetic subtypes (Owen *et al*., 2004).

In addition to the peptide inhibitors targeting gp120, several charged polymer molecules are also being evaluated as antiviral agents. Several of these include Pro-2000, cellulose sulfate, and carrageenan. These large polyanion compounds nonspecifically bind to charged residues on the viral envelope and interfere with virion binding to the cell surface (Klasse, 2006; Lederman *et al*., 2006). Since these agents cannot be delivered orally and are poorly adsorbed, they are not candidates for traditional therapy but are being investigated as topical microbicides to prevent HIV transmission.

Although the gp120 glycoprotein remains an attractive target for antiviral intervention, only limited success has been achieved in the development of orally available small molecule inhibitors. Crystallographic studies have revealed structurally conserved features on the gp120 core that could potentially act as binding sites for a small molecule (Kwong *et al*., 1998; Rizzuto and Sodroski, 2000). However, access to these cryptic-binding sites may be hampered by the presence of the variable loops, abundant glycosylation, and the genetic diversity of gp120. Despite these obstacles, few small molecule inhibitors of gp120 have been identified. The first of these is BMS 378806 which was discovered by researchers at Bristol-Myers Squibb (Lin *et al*., 2002). This compound binds directly to gp120, putatively at or near the Phe-43 cavity in the gp120 core (Kong *et al*., 2006; Madani *et al*., 2004). It is believed to act by restricting the gp120 conformational changes required for gp41-mediated fusion to occur (Si *et al*., 2004). BMS 378806 showed potent antiviral activity against both CCR5- and CXCR4-tropic clade B viruses. However, its activity against viruses from Clades A, C, and D was significantly reduced and little or no activity was observed against viruses from Clades F and G or from Group O. In addition, resistance to BMS 378806 developed rapidly *in vitro* and required only a few amino acid changes in gp120 (Guo *et al*., 2003; Lin *et al*., 2003). A follow-on compound to BMS 378806, known as BMS 488043, showed an improved spectrum of antiviral activity and has advanced to clinical trials. In a Phase Ib trial, this compound was able to achieve mean viral load reductions of greater than 1 log after two weeks of dosing, thus demonstrating clinical proof-of-concept for this new class of inhibitors (Kadow *et al*., 2006). Although these results are encouraging, further development of gp120-binding molecules with broad spectrum potency and favorable pharmaceutical properties will be needed to validate the long-term efficacy of this class of compounds.

B. Targeting CD4

Early in the course of HIV research, it was discovered that antibodies against CD4 could effectively inhibit HIV infection (Klatzmann *et al*., 1990). In animal studies, administration of anti-CD4 antibodies can lead to

depletion of CD4-positive cells and immunosuppression, an effect not desirable for HIV therapy (Sriram *et al*., 1988; Vollmer *et al*., 1987). However, researchers at Tanox, Inc. are developing a novel anti-CD4 antibody, TNX-355, which inhibits HIV-1 infection in the absence of immunosuppression (Burkly *et al*., 1992; Reimann *et al*., 1993). TNX-355 specifically binds to the D2 domain of CD4 and acts by inhibiting a post gp120-CD4-binding step in the infection process. This antibody is broadly active against viruses from different genetic clades and with different coreceptor tropism. In a Phase II trial, doses of 10 mg/kg and 15 mg/kg administered twice weekly with an optimized background regimen produced sustained viral load reductions in patients dosed for 48 weeks (Lalezari *et al*., 2006). These results support the further development of TNX-355 for HIV therapy and additional clinical studies are planned.

IV. Chemokine Receptors in HIV Infection

In 1996, several laboratories independently discovered two cellular cofactors or coreceptors that are required for HIV infection. The first coreceptor, initially named "fusin" was identified by Berger and colleagues at the National Institutes of Health (NIH) (Feng *et al*., 2000). This receptor was found to be a member of the seven-transmembrane, G-protein–coupled receptor (GPCR) family and was later renamed CXCR4 (Bleul *et al*., 1996). Expression of CXCR4 into CD4-positive cells rendered these cells susceptible to infection by T-cell line tropic viruses but not by viruses known to infect primary T cells and macrophages (M-tropic viruses). Shortly after the identification of CXCR4, several other groups published the discovery of a second chemokine receptor, CKR-5 or CCR5 that could function as a coreceptor for M-tropic HIV-1 isolates but not for T-tropic isolates (Alkhatib *et al*., 1996; Deng *et al*., 1996; Dragic *et al*., 1996). Both CCR5 and CXCR4 are members of the chemokine receptor family that play an important role in immune cell homing and chemotaxis during inflammatory responses. CXCR4 is known to bind and signal in response to the chemokine CXCL12 (SDF-1) (Bleul *et al*., 1996; Oberlin *et al*., 1996) and CCR5 binds several CC chemokines including, CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Samson *et al*., 1996).

Prior to the identification of the CCR5 and CXCR4 coreceptors, the cellular tropism of HIV-1 isolates was defined as either T-cell line tropic (T-tropic) or macrophage tropic (M-tropic), based on the type of cells a virus was capable of infecting. It is now clear that the viral tropism can be explained by differential expression of CCR5 and CXCR4 in these cell types. Currently viral tropism is defined as the preference of a virus to bind and mediate infection via either CCR5 alone (R5-tropic), CXCR4 alone (X4-tropic), or in some cases both CCR5 and CXCR4. Viruses in this last

category are known as dual-tropic or R5/X4 tropic and often consist of a mixture of R5- and X4-tropic viruses. Although some viral envelope genes do demonstrate the ability to utilize both receptors, these are relatively rare and may represent transitional viruses that are evolving from CCR5 to CXCR4 tropism (Pastore *et al.*, 2006).

During the course of HIV infection, R5-tropic viruses are preferentially transmitted and predominate in the early years of the disease. As HIV infection progresses, X4-tropic viruses emerge in about 50% of patients (Connor *et al.*, 1997; Wilkin *et al.*, 2007). The appearance of X4-tropic strains is often associated with a more rapid loss of CD4 cells and disease progression; however, it is unclear whether X4-tropic viruses are the cause or a consequence of this disease progression. Regardless, this observation may impact how tropism and efficacy are monitored during treatment with inhibitors that target these coreceptors in the near future.

V. Targeting Coreceptor Binding

Following identification of CCR5 and CXCR4 as viral coreceptors, researchers quickly recognized that these molecules could be targeted for antiviral therapy. Even prior to the discovery of CCR5, Cocchi *et al.* (1995) reported that the CC chemokines, MIP-1α, MIP-1β, and RANTES, all ligands of CCR5, could inhibit HIV infection *in vitro*. In addition, antibodies directed against CXCR4 were capable of inhibiting HIV infection (McKnight *et al.*, 1997). The most compelling evidence, however, came from a study by Liu *et al.* (1996), who identified the presence of a 32-base pair deletion (delta-32) in the CCR5 gene from a small cohort of individuals who remained HIV seronegative, despite being exposed to HIV-1 infected partners. Analysis of lymphocytes from these individuals revealed a complete lack of functional CCR5 on the cell surface. In addition, cells from these individuals were refractory to infection by R5-tropic HIV-1 strains but susceptible to X4-tropic virus infection. Furthermore, the lack of functional CCR5 was not associated with any apparent immunologic defects in these individuals. The impact of the delta-32 mutation on HIV infection was further confirmed in a larger population study that showed significantly slower disease progression in individuals heterozygous for the delta-32 CCR5 mutation relative to individuals homozygous for the wild-type CCR5 allele (Dean *et al.*, 1996). Together, these findings provided strong evidence that targeting the HIV–coreceptor interactions is a valid strategy to prevent and treat HIV infection.

A. CXCR4 Inhibition

The CXCR4 receptor is expressed on lymphocytes as well as on a wide range of other tissues in the body including, neurons, microglia,

megacaryocytes, endothelial cells, and epithelium (Delezay *et al*., 1997; Feil and Augustin, 1998; Lavi *et al*., 1997; Wang *et al*., 1998). To date, the only known ligand for CXCR4 is stromal cell-derived factor 1 (SDF-1) or CXCL12 (Bleul *et al*., 1996; Oberlin *et al*., 1996). Unlike CCR5, CXCR4 plays an essential role in early gestation by regulating cardiac and CNS development, stem cell homing, and angiogenesis. Knockout mice lacking either CXCR4 or its ligand CXCL12 die before birth and exhibit multiple cardiac, hematopoietic, and neurological defects (Ma *et al*., 1998; Zou *et al*., 1998). Therefore, blocking CXCR4 during embryonic development would be undesirable; however, the effects of CXCR4 blockade in adults are less clear and require further investigation.

1. Peptide Inhibitors of CXCR4

The first CXCR4 inhibitor to be tested in the clinic was a small positively charged 9-amino acid peptide known as ALX40-4C (Doranz *et al*., 2001). Although the mechanism of action of ALX40-4C was unknown at the time of the study, subsequent characterization revealed that this peptide binds to the second extracellular loop of CXCR4 and can block X4-tropic HIV infection *in vitro*. Although ALX-40C failed to show clinical efficacy, it was found to be safe and generally well tolerated in the study of population, a finding that encouraged further development of more potent agents. Several other CXCR4-specific peptides include, T-22, a small peptide isolated from a horseshoe crab and its derivative known as T-140 (Murakami *et al*., 1997; Tamamura *et al*., 1998). Both of these peptides have potent activity against HIV in the laboratory; however, they have not yet been advanced to proof-of-concept in the clinic.

2. Small Molecule CXCR4 Inhibitors

One hurdle to the development of peptide-based therapeutics for HIV therapy is the requirement of frequent injection for the antiviral agent. Therefore, orally available small molecules drugs that can be taken once or twice daily are more desirable for chronic, long-term HIV therapy. To date, several small molecule antagonists of the CXCR4 receptor have been identified that show potent *in vitro* antiviral activity (Fig. 2A). Some of these compounds include, AMD3100, AMD070, KRH 1636, and KRH 3140. Among these, the bicyclam AMD3100 was the first small molecule CXCR4 antagonist to be tested clinically (Hendrix *et al*., 2000, 2004; Schols, 2004). Although this molecule is not orally available and is administered by infusion, patients who received AMD3100 demonstrated a reduction in their X4-tropic virus population. However, the total viral load declines in the AMD3100 treatment group did not reach the predetermined study endpoints and the study was stopped. One patient in this study who was found to have only X4-tropic viruses did achieve a viral load decrease of 0.9 $\log_{10}$ following AMD3100 dosing, providing proof-of-concept for this

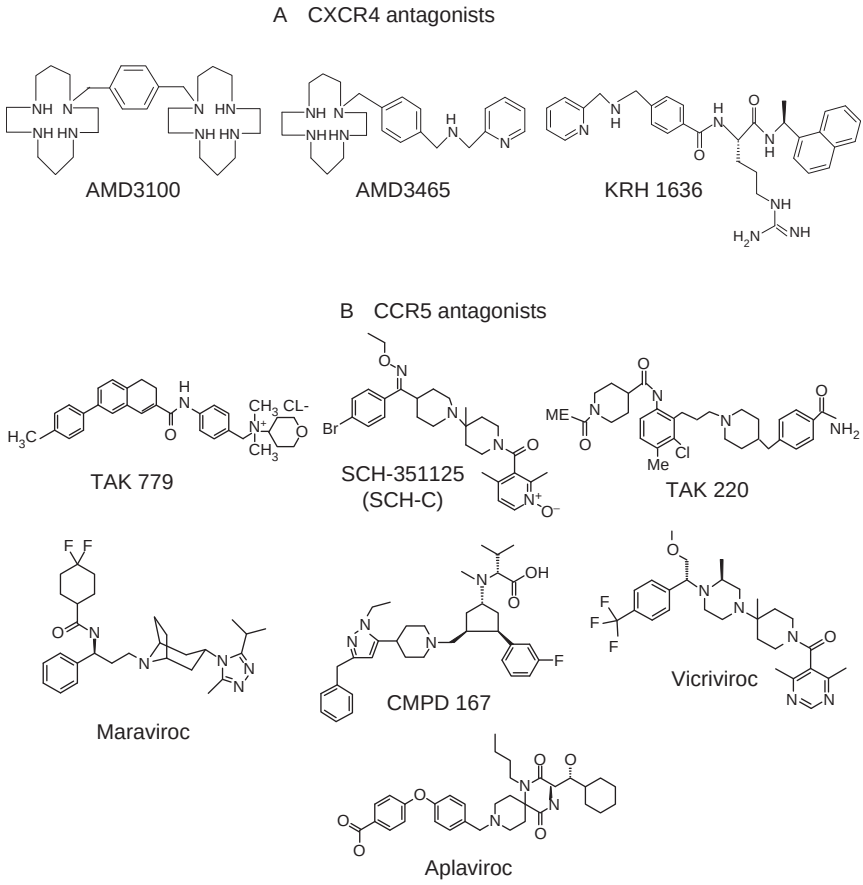


FIGURE 2 Structure of (A) CXCR4 and (B) CCR5 small molecule antagonists.

target. Interestingly, in this study, a significant increase in circulating CD4 cells was noted in patients receiving AMD3100 despite the lack of antiviral response. This effect can be attributed to disruption of the CXCR4-SDF-1 interaction that regulates lymphocyte and stem cell homing and trafficking. AMD3100 competes with SDF-1 expressed on vascular endothelial cells, which in turn causes lymphocytes and stem cells to be released from the endothelium and enter the blood stream (Devine *et al*., 2004). Although AMD3100 is no longer in development for HIV therapy, it is currently being developed as an agent to enhance stem cell mobilization and harvesting for transplantation (Broxmeyer *et al*., 2005; Cashen *et al*., 2007).

Another CXCR4 antagonist that has entered clinical trials is also a small molecule known as AMD070. This compound shows good antiviral potency *in vitro* with EC_{50} values ranging between 1 and 10 nM and has the added

advantage of oral bioavailability (Schols, 2004). Preliminary data from early clinical trials showed that twice daily dosing of AMD070 for 10 days significantly decreased CXCR4-tropic viruses in 4 of 9 patients (Moyle *et al*., 2007). Although these early results are encouraging, additional safety and long-term efficacy studies will be required for this class of compounds.

B. Targeting CCR5 for HIV Therapy

1. Peptide Inhibitors of CCR5

The first synthetic CCR5 inhibitors described were derivatives of the beta chemokine, RANTES (CCL5). Aminooxypentane-RANTES (AOP-RANTES) was synthesized by chemically modifying the N-terminal region of RANTES with an aminooxypentane group (Simmons *et al*., 1997). This molecule binds CCR5 with higher affinity and is more effective at CCR5 down modulation compared with unmodified RANTES (Mack *et al*., 1998; Signoret *et al*., 2000). Other RANTES derivatives include NNY-RANTES and PSC-RANTES. These peptide agonists inhibit an HIV infection by a combination of steric inhibition of gp120 binding as well as by inducing CCR5 down modulation at the cell surface (Mack *et al*., 1998). Both NNY-RANTES and PSC-RANTES bind CCR5 with about the same affinity as AOP-RANTES but are much more effective at internalizing and retaining CCR5 in the cytoplasm and, hence, have greatly enhanced antiviral activity (Hartley *et al*., 2004; Pastore *et al*., 2003; Sabbe *et al*., 2001). Although these modified chemokines have proven effective in animal models of infection (Lederman *et al*., 2004), their clinical development may be limited by a requirement for parenteral or topical administration and high manufacturing costs.

2. Antibody Inhibitors of CCR5

Although therapeutic antibodies are costly to manufacture and require parenteral administration, they do provide several advantages over traditional oral therapies. These include low toxicity, high specificity, and long half-life. Presently, several CCR5-specific humanized monoclonal antibodies are being developed for HIV therapy. These include Pro-140 from Progenics and HGS004 and HSG101, both from Human Genome Sciences. Pro-140 binds to a complex epitope formed by the N-terminus and the second extracellular loop of CCR5 (Olson *et al*., 1999; Trkola *et al*., 2001). Pro-140 effectively inhibits a broad range of HIV isolates at nanomolar concentrations but does not inhibit chemokine binding or receptor function at these concentrations (Trkola *et al*., 2001). In Phase I safety and pharmacokinetic study, the half-life of Pro-140 in plasma was 2–3 weeks and CCR5-positive cells remained coated with antibody for up to 60 days following a single infusion of 5 mg/kg of antibody (Lalezari *et al*., 2006).

Another CCR5-specific humanized antibody in development is HGS004. In a Phase I efficacy and safety study, doses ranging between 8 and 20 mg/kg of HSG004 resulted in mean viral load reduction of >1 log, following a single infusion. HGS is also developing a second antibody, HGS101 that reportedly had fivefold greater potency against a broader range of viruses (Lalezari *et al.*, 2006). Additional safety and long-term efficacy data will be needed to assess the role of antibody-based therapeutics in HIV therapy.

3. Small Molecule CCR5 Antagonists

As previously mentioned, CCR5 belongs to the seven-transmembrane GPCR family, a group that is amenable to targeting by small molecule drugs (Schyler and Horuk, 2006). As such, a growing number of small molecule antagonists of CCR5 have been identified and are being explored in the clinic (Table I). The structures of several of these are shown in Fig. 2B. The first small molecule CCR5 antagonist reported was TAK 779 (Baba *et al.*, 1999). *In vitro*, TAK 779 was shown to preferentially bind to CCR5 and inhibit ligand binding, CCR5 receptor activation, signaling, and cellular chemotaxis. More importantly, this compound potently blocked infection of primary cells by R5-tropic (but not X4-tropic) viruses at concentrations in the low nanomolar range. Although its profile *in vitro* was promising, TAK 779 was found to be poorly bioavailable and was not developed further. A second small molecule CCR5 antagonist known as SCH-C (or SCH 351125) demonstrated similar *in vitro* antiviral potency and antagonistic activity in preclinical studies compared with TAK 779. However, SCH-C also showed good oral bioavailability in animals, which allowed it to advance into clinical trials (Strizki *et al.*, 2001). In a small monotherapy study, 25, 50, and 100 mg of SCH-C was administered twice daily resulted in a dose-dependent decrease in viral load up to $\sim$1 $\log_{10}$ over a 10-day dosing period (Maeda *et al.*, 2004b). This study demonstrated the first clinical proof-of-concept for the utility CCR5 antagonists as antiviral agents. However, SCH-C is no longer in development because of undesirable cardiac side effects observed in the higher dose groups (Maeda *et al.*, 2004b). Following in the footsteps of SCH-C, a number of other small molecule CCR5 antagonists have entered preclinical and clinical development including maraviroc (UK-427857), aplaviroc (GW 873140), vicriviroc (SCH 417690), INCB 9471, TAK 220, and TAK 652 (Dorr *et al.*, 2005; Maeda *et al.*, 2004a; Seto *et al.*, 2006; Strizki *et al.*, 2005). Early phase clinical data are promising for this class of compounds with maraviroc, aplaviroc, and vicriviroc, all demonstrating viral load reductions of >1.5 logs following 10–14 days of dosing in patients harboring only R5-tropic viruses (Fatkenheuer *et al.*, 2005; Lalezari *et al.*, 2005; Schurmann *et al.*, 2007). While aplaviroc is no longer in development, larger Phase II/III studies, with both vicriviroc and maraviroc, demonstrated good safety profiles and sustained viral load responses in treatment-experienced patients dosed for 24 weeks or longer (Gulick *et al.*, 2006; Lalezari, *et al.*, 2007).

TABLE I Inhibitors of HIV Entry

Stage of inhibition	*Target*	*Agent*	*Description*	*Mechanism of action*
Attachment	gp120	BMS 378806	Small molecule	Induce conformation changes in gp120 that prevent attachment to cell surface receptors
		BMS 488043	Small molecule	
		IC9564	Small molecule	
		Cyanovirin-N (CNV-N)	Polypeptide from cyanobacteria	Inhibit gp120-CD4 binding
		Pro-542	Tetrameric CD4-IgG fusion protein	
		Retrocyclin-2	18aa theta defensin	
		Pro-2000	Sulfated polyanion	
		cellulose sulfate	Sulfated polyanion	
		Carregeenan	Polyanion from seaweed	
	CD4	TNX-355	Humanized monoclonal antibody	Inhibit gp120-CD4 binding
		NSC 13778	Small molecule	
Coreceptor binding	CCR5	Maraviroc	Small molecule	Stabilize conformation of CCR5 not recognized by gp120
		Vicriviroc	Small molecule	
		Aplaviroc	Small molecule	
		ICBN9471	Small molecule	
		TAK 220	Small molecule	
		TAK 652	Small molecule	
		CMPD 167	Small molecule	
		Pro-140	Humanized monoclonal antibody	Steric inhibition of gp120 binding to CCR5
		HGS004	Humanized monoclonal antibody	
		HGS101	Humanized monoclonal antibody	
		AOP-RANTES	Aminooxypentane-RANTES	Internalization and sequestration of CCR5 in cytoplasm, steric inhibition at cell surface
		NNY-RANTES	N-nonanol-RANTES	
		PSC-RANTES	N-nonanoyl, thioproline, cyclohexylglycine-RANTES	

	CXCR4	AMD 3100	Small molecule	Stabilize conformation of CXCR4 not recognized by gp120
		AMD 070	Small molecule	
		KRH 1636	Small molecule	
		KRH 3140	Small molecule	
		ALX40-C	9-amino acid peptide	Inhibition of gp120-CXCR4 binding
		T-22	18-amino acid peptide	
		T-140	14-amino acid peptide	
Fusion	gp41	Enfuvirtide (T-20, Fuzeon)	36 amino acid peptide	Prevents helical bundle formation
		TRI-999	36-amino acid peptide	
		TRI-1144	38-amino acid peptide	
		5M038	Small molecule	Prevents helical bundle formation
		5M041	Small molecule	
	Lipid rafts	SP-01A	Small molecule	Reduces membrane cholesterol and membrane fluidity

One consideration for the use of CCR5 antagonists for HIV therapy is that these compounds are only active against CCR5-tropic and not CXCR4-tropic viruses. In monotherapy studies, patients with mixed-tropic viruses treated with a CCR5 antagonist had reduced or minimal antiviral responses relative to patients with R5 only viral populations (Westby *et al*., 2006). Similar findings were observed in a larger Phase II study when maraviroc was given to HIV patients with documented R5/X4 mixed infection in the context of optimized background therapy (OBT). Although viral load reductions in the maraviroc arms were not statistically different from the placebo group, a clear increase in CD4 cells counts was measured in patients receiving maraviroc (Youle, 2006). Although these early clinical results are encouraging, additional safety and long-term efficacy data are needed to more fully define the role of this class of antiviral agents in HIV therapy.

4. Binding and Mechanism of Action of Coreceptor Antagonists

Several groups have mapped the binding site of various CCR5 antagonists including TAK 779, TAK 220, SCH-C, and aplaviroc, using alanine scanning mutagenesis of CCR5 and binding or functional assays (Dragic *et al*., 2000; Maeda *et al*., 2006; Nishikawa *et al*., 2005; Seibert *et al*., 2006; Tsamis *et al*., 2003). Even though these compounds are structurally distinct (Fig. 2B), they all appear to bind within a hydrophobic pocket formed near the junction of the extracellular loops and the transmembrane domains of CCR5. A model depicting the putative binding site of one small molecule antagonist, SCH-C, is shown in Fig. 3. Mutation of specific residues within

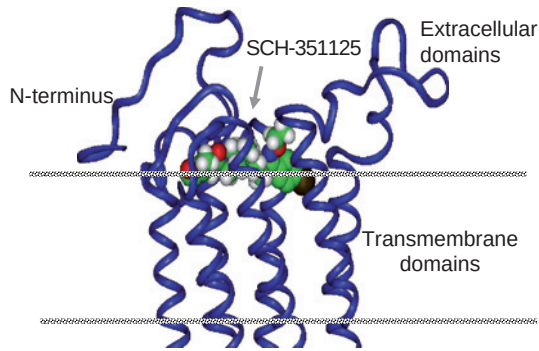


FIGURE 3 Model of a small molecule binding to CCR5. A model of CCR5 was generated based on the crystal structure of bacterial rhodopsin and a putative binding site for the small molecule antagonist SCH-351125 was modeled on the receptor based on mutagenesis and binding studies. Small molecules, such as SCH 351125, bind within a hydrophobic pocket formed within the membrane near the surface of the cells by the transmembrane domains and proximal extracellular loop regions. Mapping studies have demonstrated that individual small molecules contact different residues within the pocket and extracellular loops. This differential binding can account for different activities of the compounds on the receptor.

transmembrane domains 1, 2, 3, and 7 of CCR5 was shown to reduce the binding of SCH-C to CCR5 (Dragic *et al*., 2000; Nishikawa *et al*., 2005; Seibert *et al*., 2006; Tsamis *et al*., 2003). The most dramatic reduction in activity was seen when position E283 in TM7 was mutated to an alanine. This E283 mutation also significantly affected the binding of aplaviroc and TAK 779, suggesting that this residue is a common anchoring point for these compounds (Maeda *et al*., 2006; Nishikawa *et al*., 2005). Mutational analysis has also revealed differences in the binding interactions among the various compounds. For example, mutation of K191 at the junction of the extracellular loop 2 and transmembrane domain 5 significantly reduced binding of aplaviroc but had no effect on either TAK 779 or SCH-C. In contrast, a Y73A mutation in TM1 reduced SCH-C binding but did not affect either aplaviroc or TAK 779 binding. The binding properties of aplaviroc are somewhat unique to a receptor antagonist. While aplaviroc effectively inhibits receptor signaling, it does not inhibit binding of the ligand RANTES to CCR5 like other antagonists (Maeda *et al*., 2004a, 2006). Together these results show that while the binding sites of these small molecules overlap, they are not identical and that these differences in binding can influence the confirmation and properties of CCR5 on the cell surface.

The mechanism of action of the small molecule antagonists is quite distinct from that of the larger peptidic agonists (i.e., AOP-RANTES) and blocking antibodies. Unlike receptor agonists, these compounds do not induce downregulation of the receptor; in fact, they can inhibit CCR5 internalization induced by chemokines (Pugach *et al*., 2006). Since these molecules do not directly bind to the outer portions of the extracellular loops of CCR5, it is unlikely that they directly compete with gp120 for CCR5 binding. Rather, increasing evidence suggests that small molecule antagonists inhibit HIV infection by altering or stabilizing a conformation of the CCR5 that is unfavorable for gp120 binding (Pugach *et al*., 2006; Westby *et al*., 2007).

VI. Fusion Inhibitors

A. Targeting gp41

The final stage in the viral entry process is the fusion of the cellular and viral membranes. This process is mediated by the viral gp41 envelope glycoprotein. Following coreceptor binding, gp120 undergoes a conformational change that releases the steric hindrance on gp41, thus allowing for gp41 rearrangement and insertion of the N-terminal fusion peptide into the host cell membrane (Fig. 1). This step is followed by another conformational change in gp41 in which a six-helix bundle is formed by binding of the heptad repeat 1 region (N-terminus) to the heptad repeat 2 region (C-terminus)

within the gp41 trimer complex. This "zipping" process draws the cellular and viral membranes together to allow formation of a fusion pore (Markovic and Clouse, 2004).

The first fusion inhibitor to be approved for HIV therapy was enfuvirtide (T-20, Fuzeon), a 36-amino acid peptide designed to mimic the HR2 domain of gp41 (Wild *et al.*, 1994, 1995). Following exposure of the fusion peptide after coreceptor binding, the HR1 and HR2 domains are transiently exposed. Enfuvirtide binds to the HR1 region of gp41 and effectively prevents formation of the six-helical bundle necessary for membrane fusion. *In vitro*, enfuvirtide has broad spectrum activity and is active against both R5- and X4-tropic isolates. In clinical trials, enfuvirtide has proven effective in lowering viral loads in treatment-experienced patients when used in combination with other effective antiviral drug (Kilby *et al.*, 1998; Nelson *et al.*, 2005; Trottier *et al.*, 2005). Although enfuvirtide is generally safe and well tolerated, its use has been somewhat limited because of the requirement for twice daily injections of the drug. Therefore, efforts are underway to improve both the potency and pharmacokinetic properties of gp41-specific peptides.

To this end, several new peptides such as, TR-1144 and TR-999, have recently been described by researchers at Trimeris. These peptides show improved potency and pharmacokinetic profiles compared with enfuvirtide and TRI-1144 was selected for continued development (Lalezari *et al.*, 2006).

Crystallographic studies have identified the presence of a small pocket within the core of gp41 as a potential target for small molecule binding (Chan *et al.*, 1998). Only recently, small molecule inhibitors have been identified that are capable of inhibiting the gp41 intramolecular protein–protein interaction that occurs during bundle formation. The compounds 5M038 and 5M041 were discovered using an *in vitro* single-chain protein assay that mimics the gp41conformational change (Frey *et al.*, 2006). These compounds are believed to bind to the gp41 inner core and prevent viral fusion with IC_{50} values of about 5 μM. Additional studies will be needed to better understand how these prototypic inhibitors interact with gp41 in order to design more potent gp41-specific small molecules.

B. Inhibition of Membrane Fusion

The process of membrane fusion between the viral envelope and cell is a dynamic process that requires movement of receptors and lipids within the cell membrane. Liao *et al.* (2001, 2003) have shown that reduction of membrane fluidity by cholesterol depletion renders cells resistant to HIV infection. A group of researchers at Samaritan Pharmaceuticals has discovered an orally available small molecule entry inhibitor, SP-01A, that is believed to function by reducing intracellular cholesterol levels and

disrupting lipid rafts that facilitate in viral entry (Xu *et al*., 2003). Early phase safety and efficacy studies of SP-01A are ongoing and results should provide additional proof-of-concept for this mechanism of action in antiviral therapy.

VII. Resistance to Inhibitors of Viral Entry

One of the major challenges faced by researchers and clinicians developing novel therapies for HIV is the understanding of the mechanism by which resistance occurs and how to identify the emergence of resistance in the clinic. Characterizing the resistance pattern of entry inhibitors is no exception and is proving to be even more challenging because of the sequence heterogeneity and structural plasticity of the viral envelope proteins that mediate attachment and fusion.

In the case of the fusion inhibitor, enfuvirtide, mutations mapping to the enfuvirtide binding site on gp41 (positions 36–45) often result in reduced susceptibility (Greenberg and Cammack, 2004). However, in the clinic, mutations in other regions of gp41 have also been associated with reduced enfuvirtide susceptibility (Loutfy *et al*., 2006; Su *et al*., 2006). *In vitro* studies have shown that viruses with resistance to enfuvirtide are often less fit than wild-type viruses and they remain susceptible to coreceptor antagonists and neutralizing antibodies (Reeves *et al*., 2005). Changes in gp120 can also affect enfuvirtide susceptibility. Viral envelopes that bind with higher affinity to coreceptor and fuse more efficiently tend to have reduced susceptibility to enfuvirtide presumably due to a shorter window of access to the target sequence in the HR1 domain (Reeves *et al*., 2004).

Understanding resistance to the CCR5 and CXCR4 antagonists poses a unique challenge. Since these drugs target a cellular protein and not a viral protein, it is more difficult to predict the genotypic changes required to develop resistance. The coreceptor-binding site on gp120 is believed to involve a relatively large surface composed of the V3 loop, bridging sheet, and C4 regions (Rizzuto and Sodroski, 2000; Rizzuto *et al*., 1998). These regions form a conformational binding structure that interacts with the N-terminus and extracellular loops of the chemokine receptor. Because of the complexity of the binding interaction and genetic heterogeneity of gp120, genotypic resistance testing may not be readily possible for this class of inhibitors. Preliminary data on resistance to CCR5 antagonists reveals that while resistance can be generated *in vitro* following numerous passages, the pattern and location of mutations observed is strain specific (Baba *et al*., 2007; Trkola *et al*., 2002; Westby *et al*., 2007). Most resistant strains harbored mutations or deletions in the V3 loop, which is important for coreceptor binding; however, mutations occurring in other regions of gp120 also contribute resistance. In one case, a virus resistant to vicriviroc

was generated that did not have any mutations in the V3 loop mutation rather had multiple changes throughout gp120 and gp41 (Marozsan *et al.*, 2005). Since no genotypic test is available to assess resistance clinically, researchers must rely on phenotypic evaluations to measure susceptibility. Phenotypic resistance to reverse transcriptase and inhibitor drugs is typically quantified as a fold change in the IC_{50} dose-response curve, which is characteristic for competitive inhibitors. Unlike these inhibitors, coreceptor antagonists act allosterically and as such resistance is manifested by changes in the maximal inhibition value or "plateau" of the dose-response curve rather than a shift in IC_{50} value (Westby *et al.*, 2007). As these inhibitors advance in clinical trials, additional data on viral susceptibility and gp120 sequences changes will be needed to better develop guidelines for phenotypic susceptibility and treatment outcome.

VIII. Use Entry Inhibitors as Microbicides

The lack of an effective vaccine against HIV led researchers to investigate alternative methods to prevent HIV transmission in the hope of controlling the global epidemic. One approach being evaluated in the clinic is the use of topical microbicides to prevent transmission via mucosal surfaces. The first microbicides tested in the clinic were nonspecific surfactants that work as membrane disruptors. Agents such as, Nonoxinol-9, although active against HIV and other viruses *in vitro*, were found to cause irritation and damage of mucosal membranes and possibly enhance HIV transmission (Hillier *et al.*, 2005). This finding leads researchers to look for other less toxic and more specific agents. Sulfated polyanions, such as cellulose sulfate (Ushercell), carrageenan (Carraguard), an extract from seaweed, and Pro-2000 (Keller *et al.*, 2006), bind to charged residues on the HIV envelope protein to coat the virion and prevent viral attachment and entry (Klasse, 2006; Lederman *et al.*, 2006). *In vitro*, these agents are active against both HIV and are less cytotoxic than the detergent-based microbicides. Several of polyanionic compounds have advanced to Phase III clinical trials; however, it was recently reported that the CONRAD cellulose sulfate trial was stopped because of safety concerns (Doncel, 2007).

Antiviral compounds that specifically target the viral receptors on cells are also being explored as topical microbicides. In a macaque model of infection, Lederman *et al.* (2004) demonstrated that PSC-RANTES applied vaginally prior to viral challenge was able to protect animals from infection (Lederman *et al.*, 2004). In a similar study, Veazey *et al.* (2003) showed that a small molecule CCR5 antagonist, CMP 167, was able to protect a proportion of animals from simian-human immunodeficiency virus (SHIV) challenge and in animals that became infected, plasma RNA titers were lower in the CMPD 167-treated group. In second study, Veazey *et al.* (2005) tested

the efficacy of three antiviral compounds, a CCR5 antagonist (CMPD 167), a gp120 inhibitor (BMS-378806), and a fusion inhibitor (C52L) both alone and in combination for their ability to prevent SHIV infection in the macaque model. Each compound when administered either alone or in combination with other inhibitors was able to protect a proportion of the animals challenged. However, when viral challenge was delayed 2–12 h after treatment with CMPD 167, only about half of the animals were protected from infection, suggesting that the timing of application may be important for optimal efficacy of microbicides. While the results of these preclinical studies are encouraging, much work still needs to be done to demonstrate the safety and efficacy of entry inhibitors for prevention of HIV transmission.

References

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996). CC CKR5: A RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.

Allaway, G. P., Davis-Bruno, K. L., Beaudry, G. A., Garcia, E. B., Wong, E. L., Ryder, A. M., Hasel, K. W., Gauduin, M. C., Koup, R. A., and McDougal, J. S. (1995). Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates. *AIDS Res. Hum. Retroviruses* **11**, 533–539.

Baba, M., Nishimura, O., Kanzaki, N., Okamoto, M., Sawada, H., Iizawa, Y., Shiraishi, M., Aramaki, Y., Okonogi, K., Ogawa, Y., Meguro, K., and Fujino, M. (1999). A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc. Natl. Acad. Sci. USA* **96**, 5698–5703.

Baba, M., Miyake, H., Wang, X., Okamoto, M., and Takashima, K. (2007). Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. *Antimicrob. Agents Chemother.* **51**, 707–715.

Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**, 829–833.

Boyd, M. R., Gustafson, K. R., McMahon, J. B., Shoemaker, R. H., O'Keefe, B. R., Mori, T., Gulakowski, R. J., Wu, L., Rivera, M. I., Laurencot, C. M., Currens, M. J., Cardellina, J. H., *et al.* (1997). Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: Potential applications to microbicide development. *Antimicrob. Agents Chemother.* **41**, 1521–1530.

Broxmeyer, H. E., Orschell, C. M., Clapp, D. W., Hangoc, G., Cooper, S., Plett, P. A., Liles, W. C., Li, X., Graham-Evans, B., Campbell, T. B., Calandra, G., Bridger, G., *et al.* (2005). Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* **201**, 1307–1318.

Burkly, L., Olson, D., Shapiro, R., Winkler, G., Rosa, J., Thomas, D., Williams, C., and Chisholm, P. (1992). Inhibition of HIV infection by a novel CD4 domain 2-specific monoclonal antibody. Dissecting the basis for its inhibitory effect on HIV-induced cell fusion. *J. Immunol.* **149**, 1779–1787.

Butler, I. F., Pandrea, I., Marx, P. A., and Apetrei, C. (2007). HIV genetic diversity: Biological and public health consequences. *Curr. HIV Res.* **5**, 23–45.

Cashen, A. F., Nervi, B., and DiPersio, J. (2007). AMD3100: CXCR4 antagonist and rapid stem cell-mobilizing agent. *Future Oncol.* **3**, 19–27.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273.

Chan, D. C., Chutkowski, C. T., and Kim, P. S. (1998). Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proc. Natl. Acad. Sci. USA* **95**, 15613–15617.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., and Harrison, S. C. (2005). Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**, 834–841.

Clapham, P. R., Weber, J. N., Whitby, D., McIntosh, K., Dalgleish, A. G., Maddon, P. J., Deen, K. C., Sweet, R. W., and Weiss, R. A. (1989). Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. *Nature* **337**, 368–370.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**, 1811–1815.

Cole, A. L., Herasimtschuk, A., Gupta, P., Waring, A. J., Lehrer, R. I., and Cole, A. M. (2007). The retrocyclin analogue RC-101 prevents human immunodeficiency virus type 1 infection of a model human cervicovaginal tissue construct. *Immunology* **121**, 140–145.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997). Change in coreceptor use coreceptor use correlates with disease progression in HIV-1–infected individuals. *J. Exp. Med.* **185**, 621–628.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., *et al.* (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**, 1856–1862.

Deen, K. C., McDougal, J. S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P. J., Axel, R., and Sweet, R. W. (1988). A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature* **331**, 82–84.

Delezay, O., Koch, N., Yahi, N., Hammache, D., Tourres, C., Tamalet, C., and Fantini, J. (1997). Co-expression of CXCR4/fusin and galactosylceramide in the human intestinal epithelial cell line HT-29. *AIDS* **11**, 1311–1318.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., *et al.* (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661–666.

Devine, S. M., Flomenberg, N., Vesole, D. H., Liesveld, J., Weisdorf, D., Badel, K., Calandra, G., and DiPersio, J. F. (2004). Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J. Clin. Oncol.* **22**, 1095–1102.

Dey, B., Lerner, D. L., Lusso, P., Boyd, M. R., Elder, J. H., and Berger, E. A. (2000). Multiple antiviral activities of cyanovirin-N: Blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. *J. Virol.* **74**, 4562–4569.

Doms, R. W. (2001). Chemokine receptors and HIV entry. *AIDS* **15**(Suppl. 1), S34–S35.

Doms, R. W., and Moore, J. P. (2000). HIV-1 membrane fusion: Targets of opportunity. *J. Cell Biol.* **151**, F9–F14.

Doncel, G. (2007). "14th Conference on Retroviruses and Opportunistic Infections." Los Angeles, CA, Abstract 106LB.

Doranz, B. J., Filion, L. G., Diaz-Mitoma, F., Sitar, D. S., Sahai, J., Baribaud, F., Orsini, M. J., Benovic, J. L., Cameron, W., and Doms, R. W. (2001). Safe use of the CXCR4 inhibitor ALX40-4C in humans. *AIDS Res. Hum. Retroviruses* **17**, 475–486.

Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M., Mori, J., Rickett, G., Smith-Burchnell, C., Napier, C., Webster, R., Armour, D., *et al.* (2005). Maraviroc

(UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* **49**, 4721–4732.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667–673.

Dragic, T., Trkola, A., Thompson, D. A., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P., and Moore, J. P. (2000). A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc. Natl. Acad. Sci. USA* **97**, 5639–5644.

Fatkenheuer, G., Pozniak, A. L., Johnson, M. A., Plettenberg, A., Staszewski, S., Hoepelman, A. I. M., Saag, M. S., Goebel, F. D., Rockstroh, J. K., Dezube, B. J., Jenkins, T. M., Medhurst, C., *et al.* (2005). Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nat. Med.* **11**, 1170–1172.

Feil, C., and Augustin, H. G. (1998). Endothelial cells differentially express functional CXC-chemokine receptor-4 (CXCR-4/fusin) under the control of autocrine activity and exogenous cytokines. *Biochem. Biophys. Res. Commun.* **247**, 38–45.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.

Feng, Y., Leavitt, M., Tritz, R., Duarte, E., Kang, D., Mamounas, M., Gilles, P., Wong-Staal, F., Kennedy, S., Merson, J., Yu, M., and Barber, J. R. (2000). Inhibition of CCR5-dependent HIV-1 infection by hairpin ribozyme gene therapy against CC-chemokine receptor 5. *Virology* **276**, 271–278.

Frey, G., Rits-Volloch, S., Zhang, X.-Q., Schooley, R. T., Chen, B., and Harrison, S. C. (2006). Small molecules that bind the inner core of gp41 and inhibit HIV envelope-mediated fusion. *Proc. Natl. Acad. Sci. USA* **103**, 13938–13943.

Frost, S. D. W., Wrin, T., Smith, D. M., Pond, S. L. K., Liu, Y., Paxinos, E., Chappey, C., Galovich, J., Beauchaine, J., Petropoulos, C. J., Little, S. J., and Richman, D. D. (2005). Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. *Proc. Natl. Acad. Sci. USA* **102**, 18514–18519.

Gulick, R. M., Su, Z., Flexner, C., Hughes, M. D., Skolnick, P. R., Wilkin, T. J., Gross, R., Krambrin, K. A., Coakley, E., Greaves, W. L., Zolopa, A., Reichman, R., *et al.* (2007). Phase 2 study of the safety and efficacy of vicriviroc, a CCR5 inhibitor, in HIV-1 infected, treatment experienced patients: AIDS clinical trials group 5211. *J. Infect. Dis.* **196**, 304–312.

Greenberg, M. L., and Cammack, N. (2004). Resistance to enfuvirtide, the first HIV fusion inhibitor. *J. Antimicrob. Chemother.* **54**, 333–340.

Guo, Q., Ho, H.-T., Dicker, I., Fan, L., Zhou, N., Friborg, J., Wang, T., McAuliffe, B. V., Wang, H.-G. H., Rose, R. E., Fang, H., Scarnati, H. T., *et al.* (2003). Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. *J. Virol.* **77**, 10528–10536.

Hartley, O., Gaertner, H., Wilken, J., Thompson, D., Fish, R., Ramos, A., Pastore, C., Dufour, B., Cerini, F., Melotti, A., Heveker, N., Picard, L., *et al.* (2004). Medicinal chemistry applied to a synthetic protein: Development of highly potent HIV entry inhibitors. *Proc. Natl. Acad. Sci. USA* **101**, 16460–16465.

Hendrix, C. W., Flexner, C., MacFarland, R. T., Giandomenico, C., Fuchs, E. J., Redpath, E., Bridger, G., and Henson, G. W. (2000). Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. *Antimicrob. Agents Chemother.* **44**, 1667–1673.

Hendrix, C. W., Collier, A. C., Lederman, M. M., Schols, D., Pollard, R. B., Brown, S., Jackson, J. B., Coombs, R. W., Glesby, M. J., Flexner, C. W., Bridger, G. J., Badel, K., *et al.* (2004).

Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* **37**, 1253–1262.

Hillier, S. L., Moench, T., Shattock, R., Black, R., Reichelderfer, P., and Veronese, F. (2005). *In vitro* and *in vivo*: The story of nonoxynol 9. *J. Acquir. Immune Defic. Syndr.* **39**, 1–8.

Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., Wilson, I. A., Wyatt, R., and Kwong, P. D. (2005). Structure of a V3-containing HIV-1 gp120 core. *Science* **310**, 1025–1028.

Jacobson, J. M., Israel, R. J., Lowy, I., Ostrow, N. A., Vassilatos, L. S., Barish, M., Tran, D. N. H., Sullivan, B. M., Ketas, T. J., O'Neill, T. J., Nagashima, K. A., Huang, W., *et al.* (2004). Treatment of advanced human immunodeficiency virus type 1 disease with the viral entry inhibitor PRO 542. *Antimicrob. Agents Chemother.* **48**, 423–429.

Kadow, J., Wang, H. G., and Lin, P. F. (2006). Small-molecule HIV-1 gp120 inhibitors to prevent HIV-1 entry: An emerging opportunity for drug development. *Curr. Opin. Investig. Drugs* **7**, 721–726.

Kahn, J. O., Allan, J. D., Hodges, T. L., Kaplan, L. D., Arri, C. J., Fitch, H. F., Izu, A. E., Mordenti, J., Sherwin, J. E., and Groopman, J. E. (1990).The safety and pharmacokinetics of recombinant soluble CD4 (rCD4) in subjects with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. A phase 1 study. *Ann. Intern. Med.* **112**, 254–261.

Keller, M. J., Zerhouni-Layachi, B., Cheshenko, N., John, M., Hogarty, K., Kasowitz, A., Goldberg, C. L., Wallenstein, S., Profy, A. T., Klotman, M. E., and Herold, B. C. (2006). PRO 2000 Gel inhibits HIV and herpes simplex virus infection following vaginal application: A double-blind placebo-controlled trial. *J. Infect. Dis.* **193**, 27–35.

Kilby, J. M., Hopkins, S., Venetta, T. M., DiMassimo, B., Cloud, G. A., Lee, J. Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., Matthews, T., Johnson, M. R., *et al.* (1998). Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat. Med.* **4**, 1302–1307.

Klasse, P. J. (2006). What is the role for entry inhibitors in microbicides for blocking transmission of HIV-1 via the cervico-vaginal or rectal musosa? *J. Viral Entry* **2**, 67–72.

Klatzmann, D. R., McDougal, J. S., and Maddon, P. J. (1990). The CD4 molecule and HIV infection. *Immunodefic. Rev.* **2**, 43–66.

Kong, R., Tan, J. J., Ma, X. H., Chen, W. Z., and Wang, C. X. (2006). Prediction of the binding mode between BMS-378806 and HIV-1 gp120 by docking and molecular dynamics simulation. *Biochim. Biophy. Acta (BBA) - Proteins and Proteomics* **1764**, 766–772.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.

Lalezari, J., Thompson, M., Kumar, P., Piliero, P., Davey, R., Patterson, K., Shachoy-Clark, A., Adkison, K., Demarest, J., Lou, Y., Berrey, M., and Piscitelli, S. (2005). Antiviral activity and safety of 873140, a novel CCR5 antagonist, during short-term monotherapy in HIV-infected adults. *AIDS* **19**, 1443–1448.

Lalezari, J., Lewis, S., Subramanian, G. M., Krawiec, R., and Delmedico, M. K. (2006). Parenteral inhibitors of HIV entry in current development. *J. Viral Entry* **2**, 48–56.

Lalezari, J., Goodrich, J., DeJesus, E., Lampiris, H., Gulick, R., Saag, M., Ridgway, C., McHale, M., van der Ryst, E., and Mayer, H. (2007). Efficacy and safety of maraviroc plus optimized background therapy in viremic, ART-experienced patients infected with CCR5-tropic HIV-1: 24-week results of a Phase 2b/3 Study in the US and Canada, "*14th Conference on Retroviruses and Opportunistic Infections.*" Feb. 25–28, Los Angeles, CA, Abstract 104bLB.

Lavi, E., Strizki, J. M., Ulrich, A. M., Zhang, W., Fu, L., Wang, Q., O'Connor, M., Hoxie, J. A., and Gonzalez-Scarano, F. (1997). CXCR-4 (Fusin), a coreceptor for the type 1 human immunodeficiency virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. *Am. J. Pathol.* **151**, 1035–1042.

Lederman, M. M., Veazey, R. S., Offord, R., Mosier, D. E., Dufour, J., Mefford, M., Piatak, M., Jr., Lifson, J. D., Salkowitz, J. R., Rodriguez, B., Blauvelt, A., and Hartley, O. (2004). Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. *Science* **306**, 485–487.

Lederman, M. M., Offord, R. E., and Hartley, O. (2006). Microbicides and other topical strategies to prevent vaginal transmission of HIV. *Nat. Rev. Immunol.* **6**, 371–382.

Liao, Z., Cimakasky, L. M., Hampton, R., Nguyen, D. H., and Hildreth, J. E. K. (2001). Lipid rafts and HIV pathogenesis: Host membrane cholesterol is required for infection by HIV type 1. *AIDS Res. Hum. Retroviruses* **17**, 1009–1019.

Liao, Z., Graham, D. R., and Hildreth, J. E. K. (2003). Lipid rafts and HIV pathogenesis: Virion-associated cholesterol is required for fusion and infection of susceptible cells. *AIDS Res. Hum. Retroviruses* **19**, 675–687.

Lin, P.-F., Robinson, B., Gong, Y.-F., Ricarrdi, K., Guo, Q., Deminie, C., Rose, R., Wang, T., Meanwell, N., Yang, Z., Wang, H., Zhang, T., *et al.* (2002). Identification and characterization of a novel inhibitor or HIV Entry-I: Virology and resistance, *9th Conference on Retroviruses and Opportunistic Infections*, Feb. 24–28, Seattle, WA, Abstract 9.

Lin, P.-F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y.-F., Wang, H.-G. H., Rose, R., Yamanaka, G., Robinson, B., Li, C.-B., *et al.* (2003). A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proc. Natl. Acad. Sci. USA* **100**, 11013–11018.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367–377.

Liu, X., Lagenaur, L. A., Simpson, D. A., Essenmacher, K. P., Frazier-Parker, C. L., Liu, Y., Tsai, D., Rao, S. S., Hamer, D. H., Parks, T. P., Lee, P. P., and Xu, Q. (2006). Engineered vaginal lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. *Antimicrob. Agents Chemother.* **50**, 3250–3259.

Loutfy, M. R., Raboud, J. M., Montaner, J. S., Antoniou, T., Wynhoven, B., Smaill, F., Rouleau, D., Gill, J., Schlech, W., Brumme, Z. L., Mo, T., Gough, K., *et al.* (2006). Assay of HIV gp41 amino acid sequence to identify baseline variation and mutation development in patients with virologic failure on enfuvirtide. *Antiviral Res.* **75**, 58–63.

Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., and Springer, T. A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA* **95**, 9448–9453.

Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signoret, N., Marsh, M., Stangassinger, M., Borlat, F., Wells, T. N., Schlondorff, D., *et al.* (1998). Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: A novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* **187**, 1215–1224.

Madani, N., Perdigoto, A. L., Srinivasan, K., Cox, J. M., Chruma, J. J., LaLonde, J., Head, M., Smith, A. B., III, and Sodroski, J. G. (2004). Localized changes in the gp120 envelope glycoprotein confer resistance to human immunodeficiency virus entry inhibitors BMS-806 and #155. *J. Virol.* **78**, 3742–3752.

Maeda, K., Nakata, H., Koh, Y., Miyakawa, T., Ogata, H., Takaoka, Y., Shibayama, S., Sagawa, K., Fukushima, D., Moravek, J., Koyanagi, Y., and Mitsuya, H. (2004a). Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 *in vitro*. *J. Virol.* **78**, 8654–8662.

Maeda, K., Nakata, H., Ogata, H., Koh, Y., Miyakawa, T., and Mitsuya, H. (2004b). The current status of, and challenges in, the development of CCR5 inhibitors as therapeutics for HIV-1 infection. *Curr. Opin. Pharmacol.* **4**, 447–452.

Maeda, K., Das, D., Ogata-Aoki, H., Nakata, H., Miyakawa, T., Tojo, Y., Norman, R., Takaoka, Y., Ding, J., Arnold, G. F., Arnold, E., and Mitsuya, H. (2006). Structural and molecular interactions of CCR5 inhibitors with CCR5. *J. Biol. Chem.* **281**, 12688–12698.

Markovic, I., and Clouse, K. A. (2004). Recent advances in understanding the molecular mechanisms of HIV-1 entry and fusion: Revisiting current targets and considering new options for therapeutic intervention. *Curr. HIV Res.* **2**, 223–234.

Marozsan, A. J., Kuhmann, S. E., Morgan, T., Herrera, C., Rivera-Troche, E., Xu, S., Baroudy, B. M., Strizki, J., and Moore, J. P. (2005). Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). *Virology* **338**, 182–199.

McKnight, A., Wilkinson, D., Simmons, G., Talbot, S., Picard, L., Ahuja, M., Marsh, M., Hoxie, J., and Clapham, P. (1997). Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a coreceptor (CXCR4) is both cell type and virus strain dependent. *J. Virol.* **71**, 1692–1696.

Moyle, G., DeJesus, E., Boffito, M., Wong, R., Coakley, E., Gibney, C., Badel, K., Calandra, G., Bridger, G., and Becker, S. (2007). CXCR4 antagonism: Proof of activity with AMD11070 "14th Conference on Retroviruses and Opportunistic Infections." Abstract 511.

Moore, J. P., Parren, P. W. H. I., and Burton, D. R. (2001). Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. *J. Virol.* **75**, 5721–5729.

Mori, T., and Boyd, M. R. (2001). Cyanovirin-N, a potent human immunodeficiency virus-inactivating protein, blocks both CD4-dependent and CD4-independent binding of soluble gp120 (sgp120) to target cells, inhibits sCD4-induced binding of sgp120 to cell-associated CXCR4, and dissociates bound sgp120 from target cells. *Antimicrob. Agents Chemother.* **45**, 664–672.

Murakami, T., Nakajima, T., Koyanagi, Y., Tachibana, K., Fujii, N., Tamamura, H., Yoshida, N., Waki, M., Matsumoto, A., Yoshie, O., Kishimoto, T., Yamamoto, N., *et al.* (1997). A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J. Exp. Med.* **186**, 1389–1393.

Nelson, M., Arasteh, K., Clotet, B., Cooper, D. A., Henry, K., Katlama, C., Lalezari, J. P., Lazzarin, A., Montaner, J. S., O'Hearn, M., Piliero, P. J., Reynes, J., *et al.* (2005). Durable efficacy of enfuvirtide over 48 weeks in heavily treatment-experienced HIV-1-infected patients in the T-20 versus optimized background regimen only 1 and 2 clinical trials. *J. Acquir. Immune Defic. Syndr.* **40**, 404–412.

Nishikawa, M., Takashima, K., Nishi, T., Furuta, R. A., Kanzaki, N., Yamamoto, Y., and Fujisawa, J.-I. (2005). Analysis of binding sites for the new small-molecule CCR5 antagonist TAK-220 on human CCR5. *Antimicrob. Agents Chemother.* **49**, 4708–4715.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J. L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J. M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., *et al.* (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833–835.

Olson, W. C., Rabut, G. E., Nagashima, K. A., Tran, D. N., Anselma, D. J., Monard, S. P., Segal, J. P., Thompson, D. A., Kajumo, F., Guo, Y., Moore, J. P., Maddon, P. J., *et al.* (1999). Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. *J. Virol.* **73**, 4145–4155.

Owen, S. M., Rudolph, D. L., Wang, W., Cole, A. M., Waring, A. J., Lal, R. B., and Lehrer, R. I. (2004). RC-101, a retrocyclin-1 analogue with enhanced activity against primary HIV type 1 isolates. *AIDS Res. Hum. Retroviruses* **20**, 1157–1165.

Pastore, C., Picchio, G. R., Galimi, F., Fish, R., Hartley, O., Offord, R. E., and Mosier, D. E. (2003). Two mechanisms for human immunodeficiency virus type 1 inhibition by N-terminal modifications of RANTES. *Antimicrob. Agents Chemother.* **47**, 509–517.

Pastore, C., Nedellec, R., Ramos, A., Pontow, S., Ratner, L., and Mosier, D. E. (2006). Human immunodeficiency virus type 1 coreceptor switching: V1/V2 gain-of-fitness mutations compensate for V3 loss-of-fitness mutations. *J. Virol.* **80**, 750–758.

Pugach, P., Marozsan, A. J., Ketas, T. J., Landes, E. L., Moore, J. P., and Kuhmann, S. E. (2006). HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. *Virology* **361**, 212–228.

Reeves, J. D., Miamidian, J. L., Biscone, M. J., Lee, F.-H., Ahmad, N., Pierson, T. C., and Doms, R. W. (2004). Impact of mutations in the coreceptor binding site on human immunodeficiency virus type 1 fusion, infection, and entry inhibitor sensitivity. *J. Virol.* **78**, 5476–5485.

Reeves, J. D., Lee, F.-H., Miamidian, J. L., Jabara, C. B., Juntilla, M. M., and Doms, R. W. (2005). Enfuvirtide resistance mutations: Impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization. *J. Virol.* **79**, 4991–4999.

Reimann, K. A., Burkly, L. C., Burrus, B., Waite, B. C., Lord, C. I., and Letvin, N. L. (1993). *In vivo* administration to rhesus monkeys of a CD4-specific monoclonal antibody capable of blocking AIDS virus replication. *AIDS Res. Hum. Retroviruses* **9**, 199–207.

Rizzuto, C., and Sodroski, J. (2000). Fine definition of a conserved CCR5-binding region on the human immunodeficiency virus type 1 glycoprotein 120. *AIDS Res. Hum. Retroviruses* **16**, 741–749.

Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A., and Sodroski, J. (1998). A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**, 1949–1953.

Sabbe, R., Picchio, G. R., Pastore, C., Chaloin, O., Hartley, O., Offord, R., and Mosier, D. E. (2001). Donor-and ligand-dependent differences in C-C chemokine receptor 5 reexpression. *J. Virol.* **75**, 661–671.

Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M. (1996). Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* **35**, 3362–3367.

Schacker, T., Coombs, R. W., Collier, A. C., Zeh, J. E., Fox, I., Alam, J., Nelson, K., Eggert, E., and Corey, L. (1994). The effects of high-dose recombinant soluble CD4 on human immunodeficiency virus type 1 viremia. *J. Infect. Dis.* **169**, 37–40.

Schols, D. (2004). HIV co-receptors as targets for antiviral therapy. *Curr. Top. Med. Chem.* **4**, 833–893.

Schooley, R. T., Merigan, T. C., Gaut, P., Hirsch, M. S., Holodniy, M., Flynn, T., Liu, S., Byington, R. E., Henochowicz, S., Gubish, E., *et al.* (1990). Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. A phase I-II escalating dosage trial. *Ann. Intern. Med.* **112**, 247–253.

Schurmann, D., Fatkenheuer, G., Reynes, J., Michelet, C., Raffi, F., van Lier, J., Caceres, M., Keung, A., Sansone-Parsons, A., Dunkle, L., and Hoffmann, C. (2007). Antiviral activity, pharmacokinetics and safety of vicriviroc, an oral CCR5 antagonist, during 14-day monotherapy in HIV-infected adults. *AIDS* **21**(10), 1293–1299.

Schyler, S., and Horuk, R. (2006). I want a new drug: G-protein-coupled receptors in drug development. *Drug Discov. Today* **11**, 481–493.

Seibert, C., Ying, W., Gavrilov, S., Tsamis, F., Kuhmann, S. E., Palani, A., Tagat, J. R., Clader, J. W., McCombie, S. W., Baroudy, B. M., Smith, S. O., Dragic, T., *et al.* (2006). Interaction of small molecule inhibitors of HIV-1 entry with CCR5. *Virology* **349**, 41–54.

Seto, M., Aikawa, K., Miyamoto, N., Aramaki, Y., Kanzaki, N., Takashima, K., Kuze, Y., Iizawa, Y., Baba, M., and Shiraishi, M. (2006). Highly potent and orally active CCR5 antagonists as anti-HIV-1 agents: Synthesis and biological activities of 1-benzazocine derivatives containing a sulfoxide moiety. *J. Med. Chem.* **49**, 2037–2048.

Shenoy, S. R., O'Keefe, B. R., Bolmstedt, A. J., Cartner, L. K., and Boyd, M. R. (2001). Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins. *J. Pharmacol. Exp. Ther.* **297**, 704–710.

Si, Z., Madani, N., Cox, J. M., Chruma, J. J., Klein, J. C., Schon, A., Phan, N., Wang, L., Biorn, A. C., Cocklin, S., Chaiken, I., Freire, E., *et al.* (2004). Small-moleculeinhibitors of HIV-1 entry block receptor-induced conformational changes in the viral envelope glycoproteins. *Proc. Natl. Acad. Sci. USA* **101**, 5036–5041.

Signoret, N., Pelchen-Matthews, A., Mack, M., Proudfoot, A. E., and Marsh, M. (2000). Endocytosis and recycling of the HIV coreceptor CCR5. *J. Cell Biol.* **151**, 1281–1294.

Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N., and Proudfoot, A. E. (1997). Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276**, 276–279.

Sriram, S., Carroll, L., Fortin, S., Cooper, S., and Ranges, G. (1988). *In vivo* immunomodulation by monoclonal anti-CD4 antibody. II. Effect on T cell response to myelin basic protein and experimental allergic encephalomyelitis. *J. Immunol.* **141**, 464–468.

Strizki, J. M., Xu, S., Wagner, N. E., Wojcik, L., Liu, J., Hou, Y., Endres, M., Palani, A., Shapiro, S., Clader, J. W., Greenlee, W. J., Tagat, J. R., *et al.* (2001). SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **98**, 12718–12723.

Strizki, J. M., Tremblay, C., Xu, S., Wojcik, L., Wagner, N., Gonsiorek, W., Hipkin, R. W., Chou, C.-C., Pugliese-Sivo, C., Xiao, Y., Tagat, J. R., Cox, K., *et al.* (2005). Discovery and characterization of vicriviroc (SCH 417690), a CCR5 antagonist with potent activity against human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* **49**, 4911–4919.

Su, C., Melby, T., DeMasi, R., Ravindran, P., and Heilek-Snyder, G. (2006). Genotypic changes in human immunodeficiency virus type 1 envelope glycoproteins on treatment with the fusion inhibitor enfuvirtide and their influence on changes in drug susceptibility *in vitro*. *J. Clin. Virol.* **36**, 249–257.

Tamamura, H., Xu, Y., Hattori, T., Zhang, X., Arakaki, R., Kanbara, K., Omagari, A., Otaka, A., Ibuka, T., Yamamoto, N., Nakashima, H., and Fujii, N. (1998). A low-molecular-weight inhibitor against the chemokine receptor CXCR4: A strong anti-HIV peptide T140. *Biochem. Biophys. Res. Commun.* **253**, 877–882.

Trkola, A., Ketas, T. J., Nagashima, K. A., Zhao, L., Cilliers, T., Morris, L., Moore, J. P., Maddon, P. J., and Olson, W. C. (2001). Potent, broad-spectrum inhibition of human immunodeficiency virus type 1 by the CCR5 monoclonal antibody PRO 140. *J. Virol.* **75**, 579–588.

Trkola, A., Kuhmann, S. E., Strizki, J. M., Maxwell, E., Ketas, T., Morgan, T., Pugach, P., Xu, S., Wojcik, L., Tagat, J., Palani, A., Shapiro, S., *et al.* (2002). HIV-1escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. *Proc. Natl. Acad. Sci. USA* **99**, 395–400.

Trottier, B., Walmsley, S., Reynes, J., Piliero, P., O'Hearn, M., Nelson, M., Montaner, J., Lazzarin, A., Lalezari, J., Katlama, C., Henry, K., Cooper, D., *et al.* (2005). Safety of enfuvirtide in combination with an optimized background of antiretrovirals in treatment-experienced HIV-1-infected adults over 48 weeks. *J. Acquir. Immune Defic. Syndr.* **40**, 413–421.

Tsai, C.-C., Emau, P., Jiang, Y., Agy, M. B., Shattock, R. J., Schmidt, A., Morton, W. R., Gustafson, K. R., and Boyd, M. R. (2004). Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models. *AIDS Res. Hum. Retroviruses* **20**, 11–18.

Tsamis, F., Gavrilov, S., Kajumo, F., Seibert, C., Kuhmann, S., Ketas, T., Trkola, A., Palani, A., Clader, J. W., Tagat, J. R., McCombie, S., Baroudy, B., *et al.* (2003). Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J. Virol.* **77**, 5201–5208.

Veazey, R. S., Klasse, P. J., Ketas, T. J., Reeves, J. D., Piatak, M., Jr., Kunstman, K., Kuhmann, S. E., Marx, P. A., Lifson, J. D., Dufour, J., Mefford, M., Pandrea, I., *et al.* (2003). Use of a small molecule CCR5 inhibitor in macaques to treat simian immunodeficiency virus infection or prevent simian-human immunodeficiency virus infection. *J. Exp. Med.* **198**, 1551–1562.

Veazey, R. S., Klasse, P. J., Schader, S. M., Hu, Q., Ketas, T. J., Lu, M., Marx, P. A., Dufour, J., Colonno, R. J., Shattock, R. J., Springer, M. S., and Moore, J. P. (2005). Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. *Nature* **438**, 99–102.

Vollmer, T., Waldor, M., Steinman, L., and Conley, F. (1987). Depletion of T-4+ lymphocytes with monoclonal antibody reactivates toxoplasmosis in the central nervous system: A model of superinfection in AIDS. *J. Immunol.* **138**, 3737–3741.

Wang, J.-F., Liu, Z.-Y., and Groopman, J. E. (1998). The alpha-chemokine receptor CXCR4 is expressed on the megakaryocytic lineage from progenitor to platelets and modulates migration and adhesion. *Blood* **92**, 756–764.

Wang, W., Cole, A. M., Hong, T., Waring, A. J., and Lehrer, R. I. (2003). Retrocyclin, an antiretroviral {theta}-defensin, is a lectin. *J. Immunol.* **170**, 4708–4716.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., *et al.* (2003). Antibody neutralization and escape by HIV-1. *Nature* **422**, 307–312.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426–430.

Westby, M., Lewis, M., Whitcomb, J., Youle, M., Pozniak, A. L., James, I. T., Jenkins, T. M., Perros, M., and van der Ryst, E. (2006). Emergenceof CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. *J. Virol.* **80**, 4909–4920.

Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G., and Perros, M. (2007). Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J. Virol.* **81**, 2359–2371.

Wild, C., Greenwell, T., Shugars, D., Rimsky-Clarke, L., and Matthews, T. (1995). The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. *AIDS Res. Hum. Retroviruses* **11**, 323–325.

Wild, C. T., Shugars, D. C., Greenwell, T. K., McDanal, C. B., and Matthews, T. J. (1994). Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. *Proc. Natl. Acad. Sci. USA* **91**, 9770–9774.

Wilkin, T. J., Su, Z., Kuritzkes, D. R., Hughes, M., Flexner, C., Gross, R., Coakley, E., Greaves, W., Godfrey, C., Skolnik, P. R., Timpone, J., Rodriguez, B., *et al.* (2007). HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS clinical trial group A5211. *Clin. Infect. Dis.* **44**, 591–595.

Xu, J., Lecanu, L., Han, Z., Yao, Z., Greeson, J., and Papadopoulos, V. (2003). Inhibition of adrenal cortical steroid formation by procaine is mediated by reduction of the cAMP-induced 3-hydroxy-3-methylglutaryl-coenzyme A reductase messenger ribonucleic acid levels. *J. Pharmacol. Exp. Ther.* **307**, 1148–1157.

Youle, M. (2006). The 16th international AIDS conference meeting report. *J. Viral Entry* **2**, 77–92.

Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595–599.

Tatiana Ilina and Michael A. Parniak

Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Inhibitors of HIV-1 Reverse Transcriptase

I. Chapter Overview

Since the identification of HIV-1 as the infectious agent leading to AIDS, the viral reverse transcriptase (RT) has been a primary focus for drug discovery and development. As of mid-2007, 16 of the 28 Food and Drug Administration (FDA)-approved drugs or single dose drug combinations used for the treatment of HIV infection are inhibitors of HIV-1 RT, highlighting the importance of RT as a therapeutic target. Presently, two classes of RT inhibitors are used clinically. Nucleoside reverse transcriptase inhibitors (NRTIs) have been used clinically in the treatment of HIV-1 infection since 1986. Eight nucleoside or nucleotide RT inhibitors are in current

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56005-9

clinical use, including zidovudine (3′-azido-3′-deoxythymidine; AZT), lamivudine (2′,3′-dideoxy-3′-thiacytidine; 3TC), and the nucleotide tenofovir disoproxil fumarate (TDF). The second class is the nonnucleoside reverse transcriptase inhibitors (NNRTIs). NNRTIs are allosteric inhibitors of RT that bind to a site close to but distinct from the RT polymerase active site. There are currently three FDA-approved NNRTIs: nevirapine, delavirdine, and efavirenz. This review describes the current clinically used RT inhibitors in some detail, outlining mechanisms of action as well as factors in HIV resistance to these drugs. In addition, some promising new pipeline RT inhibitors are discussed, including compounds directed at novel RT targets not addressed by current RT therapeutics, such as inhibitors of RT ribonuclease H (RNH) activity.

II. Introduction

Retroviruses such as HIV-1 carry their genomic information in the form of (+)RNA, but must replicate through an obligatory double stranded DNA that is integrated into the infected host cell genome. Although the conversion of retroviral RNA into DNA comprises a complex series of steps, every chemical process in this conversion is catalyzed by the viral enzyme reverse transcriptase (RT). This enzyme has no cellular counterpart, and RT has therefore been considered as an attractive target for the discovery and development of drugs for the treatment of HIV-1 infection. As of mid-2007, 16 of the 28 Food and Drug Administration (FDA)-approved drugs or single dose drug combinations used for the treatment of HIV infection are inhibitors of HIV-1 RT (Table I), highlighting the importance of RT as a therapeutic target. In this chapter, we provide an overview of the current clinically used drugs or single dose drug combinations directed at RT, as well as potential drugs in late stage Phase II or Phase III clinical trials. We also discuss a number of experimental RT inhibitors with mechanisms of action distinct from that of the current approved drugs and those in late stage trials. While these experimental inhibitors are unlikely to become therapeutics, they illustrate that HIV-1 RT is likely to remain an attractive target for the discovery and development of drugs for the treatment of HIV-1 infection.

Clinically used RT inhibitors assort into two groups (1) nucleoside RT inhibitors (NRTIs) such as zidovudine (3′-azido-3′-deoxythymidine; AZT) and lamivudine (2′,3′-dideoxy-3′-thiacytidine; 3TC) and nucleotides such as tenofovir disoproxil fumarate (TDF), and (2) nonnucleoside HIV inhibitors (NNRTIs) such as nevirapine and efavirenz. All clinically approved drugs target RT DNA polymerase activity, but RT also provides additional targets for drug discovery, including inhibitors of HIV-1 RT-associated ribonuclease H (RNH), inhibitors of RT dimerization, and inhibitors of specific RT inhibitor resistance phenotypes.

TABLE I HIV-1 RT Inhibitors Approved for Therapeutic Treatment of HIV-1 Infection (2007)

Brand name	*Generic name*	*Manufacturer*
Nucleoside reverse transcriptase inhibitors (NRTIs)		
Emtriva	Emtricitabine (FTC)	Gilead
Epivir	Lamivudine (3TC)	GlaxoSmithKline
Hivid	Zalcitabine (ddC)	Hoffmann-LaRoche
Retrovir	Zidovudine (AZT)	GlaxoSmithKline
Videx (Videx EC)	Didanosine (ddI)	Bristol Myers-Squibb
Zerit	Stavudine (d4T)	Bristol Myers-Squibb
Ziagen	Abacavir sulfate (ABC)	GlaxoSmithKline
Nucleotide reverse transcriptase inhibitors (NtRTIs)		
Viread	Tenofivir disoproxil fumarate (TDF)	Gilead
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)		
Rescriptor	Delavirdine (DLV)	Pfizer
Sustiva	Efavirenz (EFV)	Bristol Myers-Squibb
Viramune	Nevirapine (NVP)	Boehringer-Ingelheim
Single dose combination products		
Atripla	EFV + FTC + TDF	Gilead
Combivir	AZT + 3TC	GlaxoSmithKline
Epzicom	ABC + 3TC	GlaxoSmithKline
Trizivir	ABC + AZT + 3TC	GlaxoSmithKline
Truvada	TDF + FTC	Gilead

III. The Target

The conversion of retroviral genomic RNA into double-stranded DNA is complex, yet all steps in this conversion are catalyzed by the retroviral RT. Accordingly, RT must be a multifunctional enzyme. RT has two types of DNA polymerase activity. The RNA-dependent DNA polymerase (RDDP) activity of RT prepares a complementary minus strand DNA copy of the viral genomic positive strand RNA. The DNA-dependent DNA polymerase (DDDP) activity of RT enables synthesis of the plus DNA strand that is complementary to the nascent minus strand DNA synthesized by RT RDDP activity. RT also has RNH activity, necessary to degrade the RNA component of the positive strand RNA–minus strand DNA duplex initially formed by RT RDDP activity. The released minus strand DNA can then serve as template for RT DDDP activity to allow completion of retroviral double-stranded DNA that can then be integrated into the host cell genome. Each of the RT activities, DNA polymerase (RDDP and DDDP) and RNH, are essential for reverse transcription and thus are appropriate targets for antiviral intervention. However, all of the inhibitors in current clinical use target RT DNA polymerase activity. Few inhibitors of HIV RT RNH activity

have been identified and as of 2007 none are in advanced preclinical development.

A. Structure of HIV-1 RT

The gene for HIV-1 RT encodes a 66-kDa protein. However, HIV-1 RT is an obligatory heterodimer consisting of two subunits of 66 and 51 kDa (Fig. 1). The p51 subunit is derived from p66 former by HIV-1 protease-catalyzed removal of the 15-kDa C-terminal RNH domain during HIV-1 assembly and maturation (reviewed by Le Grice, 1993). The structure of the p66 subunit has been likened to a "right hand" (Kohlstaedt *et al.*, 1992), with subdomains termed "fingers" (residues 1–85 and 118–155), "palm" (86–117 and 156–237), and "thumb" (238–318), that define the polymerase domain (Fig. 1). The connection domain (residues 319–426) interacts with the nucleic acid substrate as well as participating in essential protein

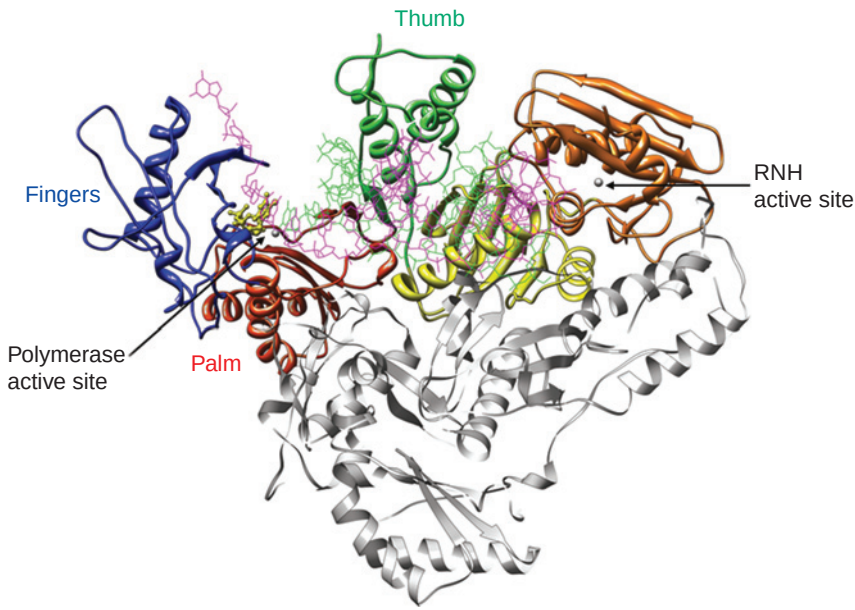


FIGURE 1 Structure of the HIV-1 reverse transcriptase (RT) p66/p51 heterodimer. The p66 subunit is rendered in color while the p51 subunit is in gray. The various subdomains of the p66 subunit are depicted in different colors: fingers (blue), palm (red), thumb (green), connection (yellow), and ribonuclease H (orange). The template strand of the bound nucleic acid is in magenta while the primer strand is green. Relative locations of the DNA polymerase and RNH active sites are indicated. The figure is based on pdb file 1RTD (Huang *et al.*, 1998) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004).

interactions between the p66 and p51 subunits. The C-terminal portion of the RT p66 subunit is the RNH domain (residues 427–565). Although the primary and secondary structural features of the p66 and p51 subunits are identical, the tertiary structures of these subunits differ substantially in the orientations of the secondary structural components. The p66 subunit is catalytically active and has an "open" conformation to accommodate the nucleic acid substrate. In contrast, the catalytically inactive p51 subunit is in a "closed" conformation. The p51 subunit is thought to play a largely structural role by acting as a scaffold for p66 (Divita *et al.*, 1995; Harris *et al.*, 1998). The p51 subunit may also play an important role in interaction with the $tRNA^{Lys3}$ primer used for the initiation of reverse transcription of the HIV-1 genomic (+)RNA (Kohlstaedt *et al.*, 1992).

The active sites for RT enzyme activities (DNA polymerase and RNH) are localized entirely within the p66 subunit. The polymerase active site is defined by a triad of catalytic aspartate residues (D110, D185, and D186) as well as several proximal residues involved in close contacts with the bound dNTP substrate (K65, R72, Y115, and Q151) (Fig. 2). The aspartates are essential for activity and mutation of any one of the triad completely eliminates RT DNA polymerase activity (Patel *et al.*, 1995). In contrast, the residues comprising components of the dNTP binding pocket can be mutated without loss of activity, although in some cases significant

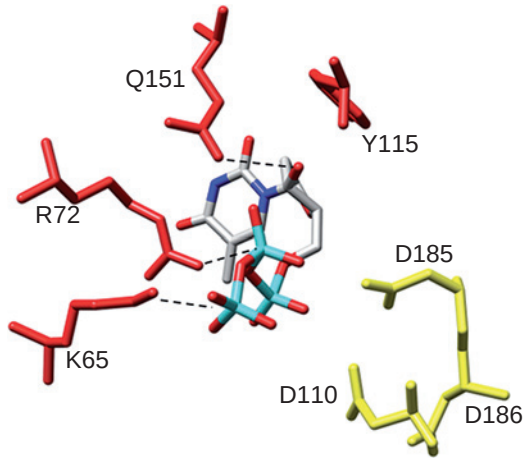


FIGURE 2 Relative positions of essential RT polymerase active site residues. The catalytic aspartates D110, D185, and D186 are in yellow, and the residues making important contacts with the bound dNTP (center) are in red. The figure is derived from pdb file 1RTD (Huang *et al.*, 1998) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004).

alterations in RT fidelity and processivity of DNA synthesis are noted (Kaushik *et al*., 1997; Sarafianos *et al*., 1995; Sluis-Cremer *et al*., 2000c). Interestingly, mutations of two of these residues, K65 and Q151, are important in HIV-1 resistance to NRTIs.

B. Mechanism of HIV-1 RT DNA Polymerase Activity

Like all DNA polymerases, HIV-1 RT DNA polymerase activity follows an ordered sequential mechanism that involves three RT mechanistic forms prior to catalysis. Free RT binds to template/primer (T/P) to form the RT–T/P binary complex. This binary complex then binds dNTP to form the RT–T/P–dNTP ternary complex. While unliganded free RT can in fact bind dNTP, the resulting RT–dNTP complex is nonproductive and does not figure in productive viral DNA synthesis (Hsieh *et al*., 1993; Kati *et al*., 1992; Majumdar *et al*., 1988; Reardon, 1992, 1993). Upon binding of the complementary dNTP to form the RT–T/P–dNTP ternary complex, HIV-1 RT undergoes a conformational change (Wu *et al*., 1993) that spatially positions the substrates to enable nucleophilic attack of the 3′-hydroxyl of the primer terminal nucleotide on the α-phosphate of the bound dNTP.

Considerable structural information relevant to the mechanism of RT activity is available as crystal structures of each of the HIV-1 RT mechanistic forms prior to catalysis are available, unliganded RT (Hsiou *et al*., 1996; Rodgers *et al*., 1995), the RT–DNA T/P binary complex (Ding *et al*., 1998; Jacobo-Molina *et al*., 1993), and the RT–DNA T/P–dNTP ternary complex (Huang *et al*., 1998). These structures provide insight into the conformational changes associated with DNA polymerization and the relative position of selected residues during the various stages of catalysis. Unliganded RT has the thumb subdomain in a "closed" position (Rodgers *et al*., 1995). The RT–T/P binary complex shows RT with the thumb moved to form a more open structure to accommodate the nucleic acid (Ding *et al*., 1998; Jacobo-Molina *et al*., 1993). Binding of dNTP to the RT–T/P complex is considered to occur in two stages. In the first, dNTP binds via protein contacts (possibly no discrimination at this stage) and in the second, the dNTP is positioned for catalysis by base-pairing with the cognate template base and closing of the fingers to form the catalytically competent closed RT–T/P–dNTP ternary complex (Huang *et al*., 1998; Sarafianos *et al*., 1999a). A rate-limiting "isomerization" or conformational change precedes actual catalysis (Hsieh *et al*., 1993); this may be related to proper spatial positioning of the dNTP to enable nucleophilic attack by the 3′-OH of the primer terminal nucleotide.

In the catalytically competent complex, the primer terminus is positioned in the so-called "priming site" or P-site. The incoming dNTP binds to the nucleotide-binding site or N-site. Nucleophilic attack by the 3′-OH of the primer terminus on the α-phosphate of the bound dNTP results in

phosphodiester-bond formation and extension of the DNA strand by one nucleotide to yield the RT–T/P_{n+1}–PPi postcatalysis ternary complex. Subsequent opening of the p66 fingers leads to release of the PPi and regeneration of the RT–T/P binary complex with the primer extended by one nucleotide. However, following incorporation of the dNMP, the primer 3′-terminus now resides in the N-site, and this primer terminus must therefore translocate to the P-site to enable incorporation of another dNTP. The efficiency of translocation determines whether the enzyme will continue polymerizing (processive synthesis) or will dissociate from the T/P (distributive synthesis) (Sarafianos *et al.*, 2002).

IV. Nucleoside RT Inhibitors

NRTIs are analogs of naturally occurring deoxyribonucleosides, but lack the 3′-hydroxyl group (Fig. 3). NRTI have been used clinically in the treatment of HIV-1 infection since 1986, and one nucleotide and seven NRTIs are in current clinical use (Table I; Fig. 3). NRTI are ineffective as administered, and must be metabolically converted by host cell enzymes to their corresponding 5′-triphosphates for antiviral activity (Furman *et al.*, 1986; Mitsuya *et al.*, 1985). The extent of phosphorylation can vary in different cell types, depending on the NRTI (Dahlberg *et al.*, 1987). The rate-limiting step in most cells is believed to be the initial phosphorylation by nucleoside kinases or, in the case of AZT, the conversion of a nucleoside monophosphate to a nucleoside diphosphate (Furman *et al.*, 1986; Gao *et al.*, 1993). There can also be substantial differences in NRTI phosphorylation in the same cell types in different patients, and this may impact on patient variability in response to antiretroviral therapy. Since NRTI are analogs of natural deoxynucleosides, there is the potential that they will be used by normal cellular DNA polymerases, with accompanying toxicity. Thus, the selectivity of NRTI use by HIV-1 RT, compared to that by cellular DNA polymerases, is an important issue in the development of NRTI as therapeutic agents.

The major limitations of nucleoside analogs include their toxicity, lack of activity in some cell types, and susceptibility to viral resistance. Toxic side effects vary but include anemia, neutropenia, peripheral neuropathy, acute pancreatitis (with ddI), and hypersensitivity syndrome (with abacavir) (Struble *et al.*, 1997).

A. Mechanisms of NRTI Inhibition

HIV-1 RT differs from cellular DNA polymerases in a number of respects in addition to using RNA as a template. RT can readily use many chemically altered analogs of the normal dNTP substrates. HIV RT also

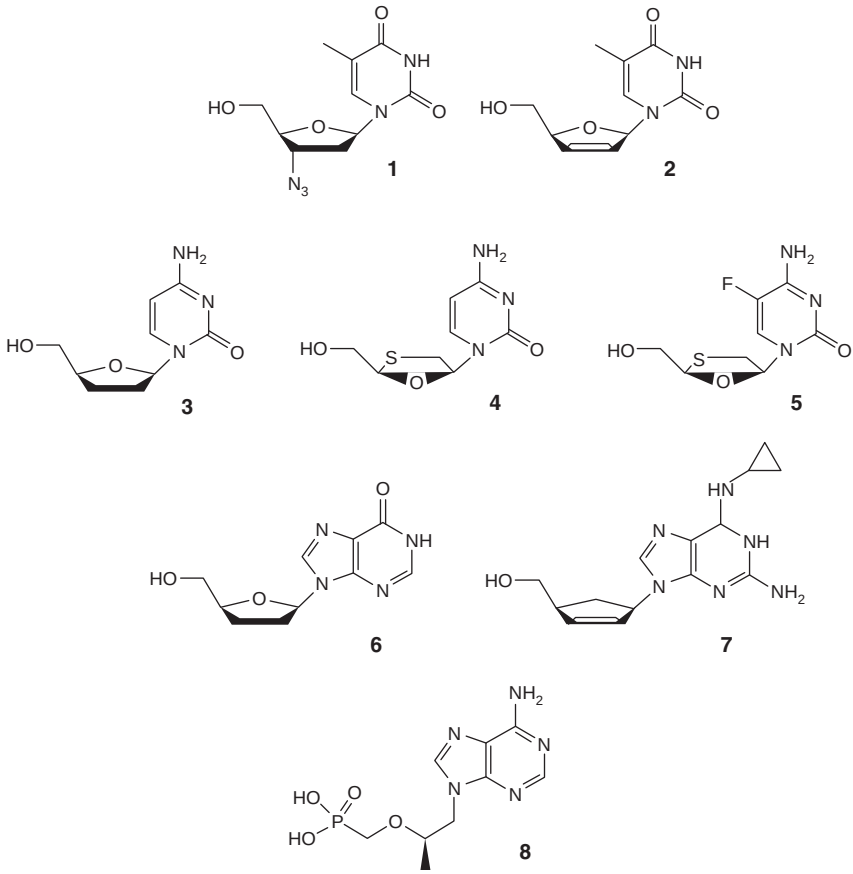


FIGURE 3 Structures of nucleoside RT inhibitors (NRTIs) approved for clinical use. **1**, Zidovudine (AZT); **2**, stavudine (d4T); **3**, zalcitabine (ddC); **4**, lamivudine (3TC); **5**, emtricitabine (FTC); **6**, didanosine (ddI); **7**, abacavir (ABC); **8**, tenofovir (TDF).

lacks a formal exonucleolytic "proofreading" activity. These characteristics are important from a pharmaceutical context, and eight of the current FDA-approved anti-HIV drugs are nucleoside or NRTIs (Table I). NRTIs are analogs of the normal dNTP substrates of DNA polymerases but lack a 3′-hydoxyl group (see Fig. 3). Once converted to the biologically active triphosphate by intracellular kinases, NRTIs inhibit RT-catalyzed DNA synthesis by two mechanisms (Sluis-Cremer *et al.*, 2000b). In the first, a given NRTI-TP competes with the corresponding natural dNTP both for recognition by RT as a substrate (binding) and for incorporation into the nascent viral DNA chain (catalysis). Thus, they are competitive inhibitors for binding and/or catalytic incorporation. However, this mechanism is only

a minor contributor to overall NRTI efficacy (Gu *et al.*, 1994b; Reardon, 1992). In the second, once an NRTI is incorporated into the nascent viral DNA chain, further viral DNA synthesis is terminated, since NRTIs lack a 3′-OH group. Chain-termination is the principal mechanism of NRTI antiviral action.

NRTIs should be "ideal" anti-HIV therapeutics. Each HIV virion carries only two copies of genomic RNA, and viral DNA synthesis involves about 20,000 RT-catalyzed nucleotide incorporations, thus providing about 5000 chances for chain-termination by any given NRTI. Since HIV-1 RT lacks a formal proofreading activity (exonuclease activity to identify and excise inappropriate nucleotide incorporation), two NRTI incorporation events should theoretically suffice to completely terminate viral DNA synthesis. However, NRTIs are not nearly so potent, for reasons that will be discussed later in this chapter. As well, although NRTI therapy is initially effective in reducing viral load in HIV patients, the viral burden inevitably rebounds despite continued therapy, due to the appearance of drug-resistant virus.

B. NRTI Approved for Clinical Use

1. Retrovir (Zidovudine; 3′-Azido-3′-Deoxythymidine; AZT)

AZT (Fig. 3, structure **1**) was first synthesized in 1964 as a potential anticancer drug, but was not further developed for human use because of concerns about toxicity. The urgent need for therapeutics prompted reassessment of nucleoside analogs as possible inhibitors, and AZT was found to have potent anti-HIV activity (Mitsuya *et al.*, 1985). AZT was the first drug approved for the treatment of HIV infection, in 1987. AZT is a thymidine analog in which the 3′-hydroxyl group has been replaced with an azido group ($-N_3$). The compound inhibits HIV replication in the 50–500 nM range (Mitsuya *et al.*, 1985). AZT is readily phosphorylated by cellular thymidine kinase to the AZT-MP. However, further phosphorylation to AZT-DP by the enzyme thymidylate kinase is difficult and rate limiting for the formation of the active antiviral agent AZT-TP (Furman, *et al.*, 1986). However, the potency of AZT-TP against HIV-1 RT is such that the low levels attained are sufficient to efficiently inhibit HIV replication. AZT has good oral bioavailability (about 60%), and shows good penetration into the central nervous system (CNS), and is therefore useful for the treatment of AIDS-related dementia. The toxicity of AZT remains a concern, with bone marrow suppression leading to anemia and/or neutropenia being the most significant dose-limiting effects of AZT therapy.

2. Videx (Didanosine; 2′,3′-Dideoxyinosine; ddI)

The 2′,3′-dideoxynucleoside didanosine (ddI; Fig. 3, structure **6**) (Mitsuya and Broder, 1988) was approved for clinical use in 1991. The active

intracellular antiviral metabolite of ddI is ddATP. Didanosine is very labile in gastric acid, and is generally administered as an enteric coated formulation (Videx EC). Oral bioavailabilty of ddI is rather less than that of AZT. Didanosine is often used in combination with stavudine plus nevirapine, or lamivudine plus nevirapine, combinations that provide a reasonably safe and effective antiviral regimen (Reliquet *et al.*, 1999).

3. Hivid (Zalcitabine; 2′,3′-Dideoxycytidine; ddC)

Zalcitabine (Fig. 3, structure **3**) (Mitsuya and Broder, 1988) was approved for clinical use in 1992. Zalcitabine is less potent than AZT, but is effective against AZT-resistant strains of HIV-1 (Jablonowski, 1995). Zalcitabine has good oral bioavailability, but absorption is significantly diminished when administered with food. The drug is readily converted to the active triphosphosphate in most cell types. Peripheral neuropathy is often associated with ddC use (Simpson and Tagliati, 1995), and ddC is no longer recommended for use in treatment regimens for antiretroviral naive patients.

4. Zerit (Stavudine; 2′,3′-Didehydro-2′,3′-Dideoxythymidine; d4T)

Stavudine (Fig. 3, structure **2**) (Lin *et al.*, 1987) was the fourth NRTI approved for clinical use in 1994. Like AZT, d4T is a thymidine analog and undergoes metabolic activation by the same sequential action of thymidine kinase and thymidylate kinase as does AZT. However, d4T-MP is a significantly better substrate than AZT-MP for thymidylate kinase, and thus higher cellular levels of the active d4T-TP are attained. Stavudine is used in combination with other antiretroviral agents but not with AZT, due to antagonism in the phosphorylation activation pathway (Riddler *et al.*, 1995). Additionally, since d4T is active against AZT-resistant HIV strains, the drug can be useful for the treatment of patients who have received prolonged previous treatment with AZT.

5. Epivir (Lamivudine; (−)-β-L-2′,3′-Dideoxy-3′-Thiacytidine; 3TC)

Like ddC, 3TC is a 2′,3′-dideoxycytidine nucleoside, but with an oxathiolane ring (Fig. 3, structure **3**) of opposite conformation to the deoxyfuranose ring of ddC. Lamivudine is about 10-fold less potent against HIV than AZT (Soudeyns *et al.*, 1991). The anti-HIV inhibitory concentration (IC_{50}) values for 3TC in different cell types range from 4 nM to 0.67 μM (Coates *et al.*, 1992), possibly due to differences in intracellular formation of the active 3TCTP. 3TC was approved for clinical use in 1995, and is quite well tolerated with minimal side effects at clinical doses. A major concern is the rapidity with which HIV-1 develops resistance to 3TC. However, the compound is generally administered in single dose combinations with AZT (Combivir) or with abacavir plus AZT (Trizivir), combinations that provide extended antiviral effect.

6. Ziagen (Abacavir; (1S, 4R)-4-[2-Amino-6(Cyclopropylamino)-9H-Purin-9yl]-2-Cyclopentene-1-Methanol; ABC)

Abacavir (Fig. 3, structure 7) was approved by the FDA in 1998, for use in combination with other antiretroviral agents for the treatment of HIV-1 infection in adults and children. ABC is the only guanosine analog thus far approved for therapy of HIV infection, and the compound also differs from other NRTI in that it is a carbocyclic nucleoside analog. ABC shows similar potency to AZT in human peripheral blood lymphocytes, shows minimal cross-resistance with AZT and other NRTI, and provides synergistic antiviral activity in combination with AZT and nevirapine in MT4 lymphocytoid cells (Daluge *et al.*, 1997). ABC is frequently administered in single dose combinations with 3TC (Epzicom), or with 3TC plus AZT (Trizivir).

ABC has a unique activation pathway (Faletto *et al.*, 1997). ABC is not a substrate for any of the cellular nucleoside kinases. Instead, the enzyme adenosine phosphotransferase catalyzes the formation of ABC monophosphate (ABC-MP). ABC-MP undergoes elimination of the 6-aminocyclopropyl group, by a specific cytosolic deaminase enzyme, to form carbovir monophosphate (CBV-MP). Interestingly, ABC itself is not a substrate for this deaminase. The active antiviral, CBV-TP, is formed via CBV-DP, by the action of normal cellular nucleoside kinases. This unique activation pathway enables ABC to overcome the pharmacokinetic and toxicological deficiencies of CBV, while maintaining potent and selective anti-HIV activity (Faletto *et al.*, 1997).

Abacavir therapy can lead to several side effects including GI disturbances, rash, and fatigue. The most serious side effect is a hypersensitivity that develops in up to 5% of the treated patients. Hypersensitized patients may no longer receive ABC therapy, since subsequent administration leads to serious, even fatal, reactions.

7. Emtriva (Emtricitabine; (−)-β-L-2′,3′-Dideoxy-3′-Thia-5-Fluorocytidine; FTC)

Emtricitabine (Fig. 3, structure 5) is identical to 3TC but for the addition of a fluorine substituent at position 5 of the cytosine ring. Emtricitabine was approved by the FDA in 2003. The compound appears to be more potent than 3TC *in vitro* but has similar cross-resistance to 3TC (Richman, 2001). Emtricitabine seems to be well tolerated, shows synergy with other antivirals, and has a long intracellular half-life. Combinations of FTC with 3TC and d4T (at standard dosages) or with protease inhibitors provide significant suppression of HIV replication (Bang and Scott, 2003). FTC is also available as single dose combinations with the nucleotide RT inhibitor tenofovir dipivoxil fumarate (TDF) (Truvada) or with TDF plus the non-NRTI efavirenz (Atripla).

8. Viread (Tenofovir; 9-[2-(Phosphonomethoxy) Propyl]Adenine; TDF)

Tenofovir (Fig. 3, structure 8) is an acyclic nucleoside phosphonate, in which the sugar moiety is replaced by an acyclic alkyl side chain linked to a phosphonate group. In nucleoside phosphonates, the labile 5′-phosphodiester bond of normal nucleotides is replaced with a stable carbon–phosphorous linkage. Nucleoside phosphonates are thus analogs of nucleotide monophosphates. The actual administered drug is a fumaric acid salt of the bis-isopropxycarbonyloxymethyl ester derivative of tenofovir, termed as Tenofovir-DF, approved by the FDA in 2001. This lipophilic diester analog greatly enhances oral uptake of the prodrug; the ester groups are removed by cellular esterases to generate the free nucleotide phosphonate. TDF has high antiretroviral activity (Balzarini *et al.*, 1993) and retains potency against viruses resistant to many other NRTIs (Srinivas and Fridland, 1998). Early data suggested that it was relatively difficult to select mutations that confer high levels of resistance to tenofovir (Margot *et al.*, 2002), although TDF-resistant HIV variants are becoming more prevalent with increasing clinical use of the drug. As discussed previously, TDF is available in single dose combinations with FTC (Truvada) or with FTC plus efavirenz (Atripla). Tenofovir-DF is currently being studied for the treatment of 3TC-resistant hepatitis B virus in patients coinfected with HIV and HBV (Yang *et al.*, 2005).

C. Investigational NRTI

1. Apricitabine [(−)-2′-Deoxy-3′-Oxa-4′-Thiacytidine; (−)dOTC]

Apricitabine (Fig. 4, structure **9**) is a cytidine analog with an unusual "sugar" ring with both oxygen and sulfur components. The compound has excellent pharmacokinetic characteristics and is active against HIV strains resistant to other NRTIs (De Muys *et al.*, 1999; Richard *et al.*, 2000). Apricitabine is presently in Phase II clinical trials.

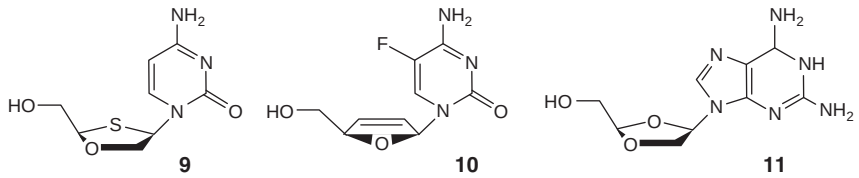


FIGURE 4 Structures of investigational NRTIs in late stage clinical trials. **9**, Apricitabine (−)dOTC; **10**, elvucitabine (L-d4FC); **11**, amdoxovir (DAPD).

2. Elvucitabine (2′,3′-Dideoxy-2′,3′-Didehydro-β-L-5-Fluorocytidine; ELV; L-d4FC)

Elvucitabine (Fig. 4, structure **10**) is an L-cytidine analog of d4T was designed to have improved activity compared to 3TC, and especially against 3TC-resistant virus. ELV has 10- to 20-fold greater antiviral activity compared to 3TC, in part due to better pharmacokinetic parameters (Dutschman *et al.*, 1998). Elvucitabine has been assessed in Phase II trials and showed potent antiviral activity against HIV resistant to 3TC and other NRTIs (Vivet-Boudou *et al.*, 2006). However, toxicity issues such as bone marrow suppression may impact future development of this NRTI.

3. Amdoxovir [(−)-β-D-2,6-Diaminopurine Dioxolane; DAPD]

Amdoxovir (Fig. 4, structure **11**) is a nucleoside analog prodrug that is deaminated by adenosine deaminase to form the anti-HIV agent (−)-β-D-dioxolane guanosine (DXG). Amdoxovir has potent activity against HIV-1 including multidrug resistant strains of the virus (Gu *et al.*, 1999; Mewshaw *et al.*, 2002). Amdoxovir was designed to overcome issues with solubility and bioavailability of DXG (Kim *et al.*, 1993), and it is rapidly absorbed and deaminated *in vivo* by adenosine deaminase to generate DXG and its active metabolite DXG-TP (Jeffrey *et al.*, 2003). Amdoxovir is currently in Phase II clinical trials.

D. HIV-1 Resistance to NRTIs

NRTI are only transiently useful as monotherapy for clinical treatment of HIV infection. Drug failure is the result of the appearance of drug-resistant variants of HIV. NRTI resistance correlates with specific mutations in HIV-1 RT. It is believed that HIV-1 undergoes facile mutation due to the low transcription fidelity of HIV-1 RT. Because of this, multiple HIV-1 variants exist *in vivo*, and drug-resistant mutants may be present at low levels before initiation of therapy (Domingo *et al.*, 1997). Under the selective pressure of anti-HIV therapy, these drug-resistant mutants gain a competitive advantage and eventually become the dominant quasispecies. The time required for this selection to occur is dependent on factors such as mutant frequency at the time of treatment initiation, the replication fitness of the mutant, and the magnitude of the selective pressure (potency of the drug).

Monotherapy is inappropriate in the treatment of HIV-infected individuals, and all patients are now treated with drug combinations. The most widely used combination therapies involve two NRTIs + a protease inhibitor, although two NRTIs + an NNRTI as well as double and triple NRTI combinations are also used. Several single dose drug combinations of NRTIs or NRTIs plus an NNRTI are currently marketed (Table I). While the use of

drug combinations delays resistance development, drug resistance inevitably occurs.

There are two major biochemical mechanisms of NRTI drug resistance (Isel *et al*., 2001; Sluis-Cremer *et al*., 2000b). The first is discrimination, that is, selective alterations in NRTI binding and/or incorporation which allow RT to exclude the NRTI, while retaining the ability to use the analogous natural dNTP substrate. The simplest example of discrimination is steric hindrance, in which the resistance mutation selectively excludes the NRTI due to unfavorable overlap of some component of the NRTI not present in the natural dNTP substrate. An example is the M184V mutation that confers high-level resistance to 3TC (Schinazi *et al*., 1993; Schuurman *et al*., 1995). Molecular modeling (Sarafianos *et al*., 1999b) suggests that the β-branched side chain of the valine substitution at position 184 makes inappropriate contact with the sulfur of the oxathiolane sugar of 3TC-triphosphate (3TCTP), preventing proper positioning of 3TCTP for catalysis. However, the mechanism for discrimination in resistance to other NRTI, such as 2′,3′-dideoxycytidine or 2′,3′-didehydro-2′,3′-dideoxythymidine, is less obvious, since in these RT must selectively ignore a structurally less rich compound (NRTI, lacking the 3′-OH) in favor of the more structurally rich dNTP analog.

In the second mechanism, mutations do not substantially alter incorporation of NRTI-TP, but instead promote the phosphorolytic removal of the chain-terminating NRTI from the 3′-end of the nascent viral DNA (Arion *et al*., 1998; Meyer *et al*., 1998, 1999). Once the NRTI is removed, viral DNA synthesis can resume. This mechanism of resistance is variously termed as pyrophosphorolysis, nucleotide excision, and primer unblocking. The hydrolytic removal of a chain-terminating NRTI requires a pyrophosphate donor, usually ATP or inorganic phyrophosphate (Arion *et al*., 1998; Meyer *et al*., 1998, 1999). Mutations that discriminate against NRTIs are generally associated with decreased enzymatic polymerase activity *in vitro*. In contrast, mutations associated with primer unblocking have much less impact on RT catalytic efficiency (Boyer *et al*., 2002a,b).

1. Mutations Associated with HIV-1 Resistance to NRTIs

Table II lists most of the important clinically seen NRTI resistance mutations and the resistance phenotype(s) associated with these. Although these phenotypes are supported by a large body of *in vitro* biochemical studies with recombinant mutant RTs, there are as yet no conclusive data to support these phenotypic resistance mechanisms at the level of HIV replication.

TABLE II Clinically Observed Mutations in Reverse Transcriptase Associated with HIV Resistance to Individual NRTI

NRTI	*Mutations*	*Resistance phenotype*
Zidovudine (AZT)	M41L, E44D, D67N, K70R, L210W, T215F/Y, K219Q	Phosphorolysis
Stavudine (d4T)	M41L, E44D, D67N, K70R, L210W, T215F/Y, K219Q	Phosphorolysis
Didanosine (ddI)	K65R, L74V	Discrimination, phosphorolysis
Zalcitabine (ddC)	K65R, T69D, L74V, M184V	Discrimination[a]
Abacavir	K65R, L74V, Y115F, M184V	Discrimination, phosphorolysis
Lamivudine (3TC)	E44D, M184V	Discrimination[a]
Tenofovir (TDF)	K65R	Discrimination, phosphorolysis
Emtricitabine (FTC)	M184V, K65R (?)	Discrimination (?)[a]

[a]NRTI based on cytidine appear to be quite refractory to the phosphorolytic excision phenotype.

a. The Thymidine Analog Mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q AZT was the first drug approved for treatment of HIV infection. Soon after initiation of AZT monotherapy, drug-resistant variants of HIV-1 became apparent. Larder and colleagues were instrumental in showing that HIV-1 resistance to AZT correlated with a number of mutations in RT, namely M41L, D67N, K70R, T215F/Y, and K219Q (Larder and Kemp, 1989). Subsequently, L210W was also found to play a role in AZT resistance. The degree of resistance correlates with the number of mutations in RT. The crystal structure of the RT–T/P–dNTP ternary complex (Huang *et al*., 1998) shows that the residues mutated to thymidine analog mutations (TAMs) are located around the dNTP binding pocket, thus suggesting a direct impact in NRTI binding (discrimination). In fact, TAMs provide only a minor effect on RT affinity for AZT-TP (Arion *et al*., 1998; Kerr and Anderson, 1997). TAMs were also proposed to alter processivity of RT DNA synthesis (Caliendo *et al*., 1996) although the relationship of increased processivity to NRTI resistance was not clear. The mechanism of AZT resistance remained uncertain for nearly 10 years following the first report of the correlation of TAMs with AZT resistance (Larder and Kemp, 1989). TAMs are now known to facilitate the phosphorolytic removal of chain-terminating NRTI-MP from the 3′-terminus of the nascent viral DNA (Arion *et al*., 1998; Meyer *et al*., 1998, 1999).

Initial studies suggested that the D67N and K70R mutations were most important in the excision of chain-terminating NRTI, and that the main effect of the T215Y/F and K219Q mutations was to induce an increase in the processivity of RT DNA synthesis to compensate for the potential detriment of an increased rate of 3′-nucleotide removal (Arion *et al*., 1998; Meyer *et al*., 1999). More recent structural and modeling studies suggest that

TAMs, especially T215F/Y, may increase the affinity of RT for the excision substrate ATP so that NRTI removal is reasonably efficient at physiologic ATP concentrations (Boyer *et al.*, 2002a; Dharmasena *et al.*, 2007). Other TAMs such as M41L and L210W may stabilize the interaction of 215Y/F with the dNTP binding pocket (Yahi *et al.*, 2000).

During active RT-catalyzed DNA synthesis, translocation of the primer 3′-terminus from the N-site to the P-site to enable incorporation of the next nucleotide may be promoted by binding of the next complementary dNTP. If, however, the primer is terminated by an NRTI, this translocation results in the formation of a "dead-end" complex of RT, T/P, and dNTP (Boyer *et al.*, 2002a; Sarafianos *et al.*, 2004). Under these conditions, the NRTI cannot undergo phosphorolytic excision, as it is trapped in the P-site, whereas phosphorolysis can only occur when the 3′-primer terminus is in the N-site. Thus, the formation of the dead-end complex prevents mutant RT from removing the terminal NRTI and resuming viral DNA synthesis. Some investigators have suggested that the bulky azido group of AZT interferes with the formation of the dead-end catalytic complex by preventing translocation of the AZT-terminated primer from the N-site to the P-site (Boyer *et al.*, 2002; Lennerstrand *et al.*, 2001). If the AZT-terminus remains in the N-site, it is more likely to undergo phosphorolytic excision, and thus ATP-dependent rescue of AZT-terminated primers is more likely to occur than rescue of primers terminated with other NRTI at the dNTP concentrations present in activated cells (Meyer *et al.*, 2000). While this observation helps explain why the effect of TAMs is most pronounced with AZT, recent studies show that the azido group is not the major determinant for excision (Sluis-Cremer *et al.*, 2005), thus the somewhat simplistic models accounting for the role of TAMs in AZT resistance (Boyer *et al.*, 2002; Lennerstrand *et al.*, 2001) need further investigation.

It is increasingly evident that TAMs provide phenotypic and clinical resistance to virtually all of the other NRTIs, not just thymidine analog inhibitors (Marcelin *et al.*, 2004). Clinical studies have shown that primer unblocking mutations, particularly mutations at position 215, interfere with the clinical response to d4T (Lin *et al.*, 1994), abacavir (Miller *et al.*, 2000), ddI (Marcelin *et al.*, 2005), and many dual-NRTI regimens (Marcelin *et al.*, 2004). The presence of four or more TAMs will typically cause >100-fold decreased susceptibility to AZT, 5- to 7-fold decreased susceptibility to abacavir, and 2- to 5fold decreased susceptibility to d4T, ddI, ddC, and tenofovir (Lennerstrand *et al.*, 2001; Miller *et al.*, 2001). In contrast, TAMs provide only low-level phenotypic resistance to 3TC and likely to other cytidine analog NRTIs.

b. M184V The M184V mutation was identified as rapidly emerging in patients receiving 3TC monotherapy (Schuurman *et al.*, 1995). M184V confers a discrimination phenotype. In wild-type RT, M184 makes contact

with both the sugar and the base of the 3′-terminal nucleotide of the primer (Huang *et al.*, 1998; Tantillo *et al.*, 1994). In the 3TC-resistant M184V mutant, the β-branched side chain of valine can also contact the sugar moiety of the bound dNTP substrate (Huang *et al.*, 1998). Molecular modeling shows that the configuration of the oxathiolane ring of 3TC is such that the β-branched side chain of the V184 residue in the mutant RT sterically contacts the large sulfur atom of the oxathiolane ring, thereby preventing the correct positioning of the bound 3TCTP to allow catalysis (Sarafianos *et al.*, 1999a).

M184V is also selected during therapy with emtricitabine, abacavir, and less commonly with ddI or ddC (Miller *et al.*, 1999; Shafer *et al.*, 1994; Shirasaka *et al.*, 1993). RT enzymes with M184V have increased fidelity (Wainberg *et al.*, 1996) and decreased processivity (Naeger *et al.*, 2001) *in vitro*, although the relevance of these properties to the resistance phenotype is unclear. M184V by itself causes high-level (>100-fold) resistance to 3TC and FTC (Tisdale *et al.*, 1993; Whitcomb *et al.*, 2002). In the absence of other drug resistance mutations, M184V leads to an ~1.5-fold reduction in ddI susceptibility and 3-fold reduction in abacavir susceptibility. In the presence of TAMs, M184V decreases susceptibility to ddI, ddC, and abacavir and increases susceptibility to AZT, d4T, and tenofovir (Miller *et al.*, 1999; Whitcomb *et al.*, 2003). Resensitization may be due to the ability of M184V to impair the rescue of chain-terminated DNA synthesis (Boyer *et al.*, 2002b; Gotte *et al.*, 2000) and may account for the slower development of AZT resistance in patients treated with combinations of 3TC and AZT or d4T (Kuritzkes *et al.*, 2000).

c. Mutations Arising in Resistance to Multiple NRTIs The M184V and TAMs mutations are correlated primarily with resistance to 3TC and AZT, respectively, although they do confer cross-resistance to other NRTI to varying degrees. Several NRTI-resistance mutations are less firmly associated with any one specific NRTI, having been identified in HIV isolates from patients treated with various NRTI. These mutations tend to localize in residues 65–75 in the fingers subdomain of RT. In the p66 subunit, several residues in this region interact with the incoming dNTP during polymerization (Boyer *et al.*, 2002a). The TAMs D67N and K70R associated with the phosphorolytic excision phenotype have already been described. A number of other mutations are also found in this region.

i. K65R. This mutation was first identified in HIV-1 cross-resistant to ddC and 3TC *in vitro* (Gu *et al.*, 1994a,b, 1995). However, this mutation was rarely found in patients treated with NRTIs and was thus considered primarily of academic interest. However, the presence of the K65R mutation has increased dramatically since the introduction of tenofovir (TDF) therapy (Valer *et al.*, 2004). K65 interacts with the γ-phosphate of the bound dNTP (Fig. 2), and the mutation K65R leads to increased discrimination by RT for

normal dNTPs compared to NRTIs, resulting in intermediate levels of resistance to ddI, ddC, 3TC, ABC, FTC, and TDF, as well as lower level resistance to d4T (Gu *et al.*, 1995; Miller *et al.*, 2000; Sluis-Cremer *et al.*, 2000b). In contrast, K65R hypersensitizes HIV-1 to AZT and does not seem to develop readily in patients receiving AZT (Whitcomb *et al.*, 2002). Like other discrimination phenotype mutations, K65R decreases HIV-1 replication capacity. It also appears to increase the replication fidelity of HIV-1 RT (Arion *et al.*, 1996).

ii. Substitutions at position 69. Alterations at position 69 in RT are the most commonly occurring NRTI resistance mutations other than TAMs and M184V. T69D was initially identified in resistance to ddC (Fitzgibbon *et al.*, 1992), but mutations at this position have been reported following treatment with each of the NRTIs (Winters and Merigan, 2001). Mutations at position 69 are not limited to amino acid substitutions, as two or more amino acid insertions are also found in resistance to NRTI. Position 69 insertions occur infrequently, in about 2% of heavily drug experienced HIV-1-infected patients (Van Vaerenbergh *et al.*, 2000). On their own, position 69 insertions provide only low-level resistance to any given NRTI. However, HIV-1 possessing 69 insertions together with T215Y/F and other TAMs in RT show high-level resistance to virtually all NRTIs (Masquelier *et al.*, 2001). Importantly, position 69 insertions can provide 20-fold resistance to TDF, the highest level of resistance to this drug reported (Miller and Larder, 2001). Insertion mutations seem to act like TAMs in that they enhance phosphorolytic NRTI excision, but they also act to destabilize dead-end complex formation, thereby enabling more facile excision of non-azido NRTIs (Boyer *et al.*, 2002; Meyer *et al.*, 2003). In addition to amino acid insertions, single amino acid *deletions* between codons 67 and 70 occur in a small number of heavily treated patients (Masquelier *et al.*, 2001). The biochemical mechanism of how these deletions enhance NRTI resistance is unknown at present.

iii. L74V. The L74V mutation arises during ddI and abacavir monotherapy (Harrigan *et al.*, 2000) and confers two- to fivefold resistance to ddI and ddC (Winters *et al.*, 1997) and two- to threefold resistance to abacavir (Harrigan *et al.*, 2000). L74V leads to AZT hypersusceptibility and is thus rarely seen in patients receiving AZT plus ddI combination therapy (Miller and Larder, 2001; Shafer *et al.*, 1994). L74 interacts with the incoming dNTP via H-bonding interactions (Huang *et al.*, 1998; Tuske *et al.*, 2004). L74V leads to decreased RT processivity *in vitro* and decreased virus replication fitness in cell culture (Diallo *et al.*, 2003).

iv. V75T. The V75T mutation leads to approximately fivefold resistance to d4T, ddI, and ddC (Lacey and Larder, 1994). Biochemical and structural modeling data suggest that mutations at this position cause drug resistance through nucleotide discrimination and possibly also through a non-ATP-mediated mechanism of primer unblocking (Selmi *et al.*, 2001). The V75T

mutation occurs only rarely *in vivo*, and generally appears in virus isolates that also have the Q151M multi-NRTI resistance mutation (Shafer, 2002).

v. Q151M complex. The Q151 mutation alone or with the generally associated mutations A62V, V75I, F77L, and F116Y (the "Q151 complex") is a multidrug resistance phenotype as this mutation complex provides relatively high-level resistance to all NRTIs. Q151 makes significant hydrogen bonding contacts with the nucleotide deoxyribose sugar including the 3′-OH (Huang *et al*., 1998; Fig. 2), thus mutation of this residue would likely to impact on the binding orientation of normal dNTP and especially dideoxynucleotide triphosphate molecules that lack the 3′-OH moiety (Huang *et al*., 1998).

Q151M apparently causes resistance to NRTI by decreasing the rate of incorporation of NRTIs relative to the natural dNTP substrates (Deval *et al*., 2002). Q151 is rare clinically, but can develop in patients receiving dual-NRTI therapy comprising ddI plus AZT or d4T (Deval *et al*., 2002; Schmit *et al*., 1998). Q151M alone induces only intermediate NRTI resistance, but addition of the mutations A62V, V75I, F77L, and F116Y leads to high-level resistance to most NRTI (Iversen *et al*., 1996; Schmit *et al*., 1998). HIV-1 isolates with Q151M appear to be antagonistic for development of mutations associated with phosphorolytic excision (TAMs, 69 insertions) (Gonzales *et al*., 2003).

vi. Y115F. The 4-OH of Y115 is positioned in the nucleotide-binding pocket such that the 2′-OH of ribonucleoside triphosphates would sterically clash. This residue is thus considered to be a "steric gate" to prevent RT from using NTPs as substrates, thereby defining RT as a DNA polymerase (Gao *et al*., 1997). Y115F is an uncommon mutation that occurs predominantly in patients receiving abacavir (Miller *et al*., 2000).

2. *NRTI Cross-Resistance*

Multiple drug combination therapies, especially those that employ single dose NRTI combinations such as Trizivir and Atripla (Table I), are highly effective at reducing viral load in HIV-infected patients. However, reliance on these single dose combinations, especially when considered with patient noncompliance, can provide a breeding ground for the development of HIV variants cross-resistant to multiple drugs. As well, addition of new NRTI to treatment regimens for patients already resistant to one or more NRTIs can lead to multidrug resistance. Indeed, many of the NRTI-resistance mutations described previously arose during sequential NRTI treatments and confer a multidrug resistance phenotype.

The most prevalent multidrug resistance pattern is the presence of TAMs, unfortunately the most common resistance mutation pattern in drug experienced patients. As described above, TAMs lead to an increased ability of HIV RT to excise the incorporated NRTI and thus allow completion of HIV DNA synthesis. Other mutations added onto TAMs, such as

69 mutations or insertions, enhance this NRTI phosphorolytic activity (Shafer, 2002).

The mutation K65R is rapidly increasing in frequency due to recent widespread use of TDF. This mutation confers cross-resistance to all NRTI except AZT (Gu *et al.*, 1994b). The Q151M complex mutation set is still rare, but provides high-level resistance to virtually all NRTIs, probably due to a discrimination mechanism. Viruses with the Q151M complex in RT seem to be quite attenuated in replication fitness however.

Despite the increasing prevalence of NRTI-resistant HIV variants, including multi-NRTI-resistant virus, nucleoside analogs will continue to play a major role in anti-HIV therapy. Current efforts are to develop analogs that are less toxic, less susceptible to viral resistance, and less dependent on specific kinases, the activity of which can vary from cell to cell.

V. Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs, as the name implies, are not analogs of dNTPs but rather comprise a large group of molecules with considerable structural diversity (De Clercq, 2005). NNRTIs are allosteric inhibitors of RT that bind to a hydrophobic region in the p66 subunit of RT termed the nonnucleoside inhibitor binding pocket (NNIBP). This site is distinct from the RT polymerase active site and NNRTIs act as noncompetitive inhibitors. NNRTIs tend to be absolutely specific for inhibition of HIV-1 RT, and generally do not inhibit RTs from HIV-2 or any other retrovirus, nor do they inhibit cellular DNA polymerases (De Clercq, 1998). NNRTIs also differ from NRTIs in that they do not require cellular metabolism for antiviral activity. There are currently three FDA-approved NNRTIs: nevirapine, delavirdine, and efavirenz (Table I; Fig. 5).

A. Mechanisms of NNRTI Inhibition

NNRTIs bind to the same region of RT, the hydrophobic NNIBP in the palm subdomain. This "pocket" is defined by residues P95, L100, V106, V108, V179, Y181, Y188, F227, W229, L234, P236, and Y318 of the RT p66 subunit with contributions from E138 of the p51 subunit (Fig. 6). Analysis of the structures of potent NNRTIs (Fig. 5) indicates several features important for interaction with the NNIBP (Tucker *et al.*, 1996). These include an aromatic ring capable of π-stacking interactions, an amide N–H able to participate in hydrogen bonding, and one or more hydrocarbon-rich regions to participate in hydrophobic contacts. The NNIBP does not exist as a specific binding pocket in RT. Rather, the side chains of the RT residues comprising the NNIBP adapt to each bound NNRTI in a specific

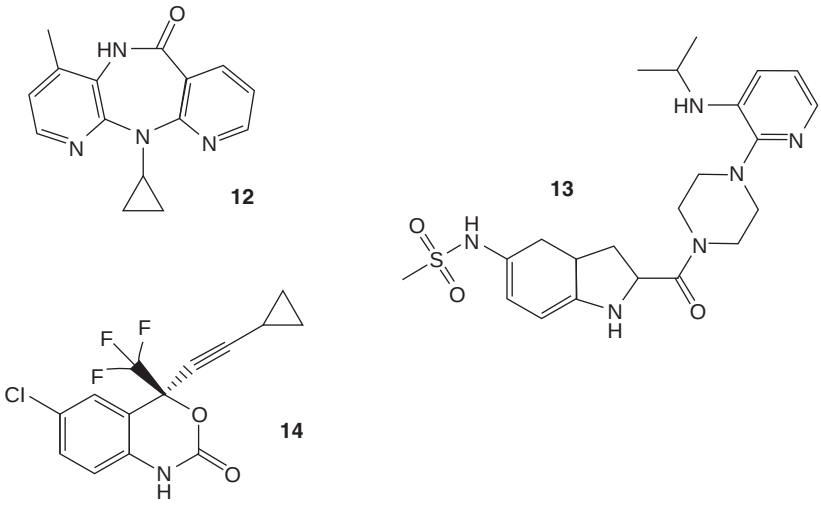


FIGURE 5 Structures of nonnucleoside RT inhibitors (NNRTIs) approved for clinical use. **12**, Nevirapine (NVP); **13**, delavirdine (DLV); **14**, efavirenz (EFV).

manner, closing down around the surface of the drug to make tight van der Waals contacts (Kroeger-Smith *et al.*, 1995).

The molecular mechanisms by which NNRTIs inhibit RT is not entirely clear, although it is expected that the same basic mechanism of inhibition will apply to all NNRTIs due to the interaction with the NNIBP. A number of possible mechanisms for NNRTI inhibition have been proposed. Conformational changes in the NNIBP upon NNRTI binding may distort the geometry of the nearby polymerase catalytic site, especially the highly conserved YMDD motif, thereby impacting on formation of the productive ternary complex. There is crystallographic (Esnouf *et al.*, 1995) and kinetic (Spence *et al.*, 1995) evidence for this possibility. In the absence of inhibitors, the rate-limiting step in RT-catalyzed DNA synthesis is a conformational change in the RT–T/P–dNTP ternary complex that enables a fast rate of nucleotide incorporation (Kati *et al.*, 1992; Majumdar *et al.*, 1988; Reardon, 1992, 1993). NNRTIs appear to change the rate-limiting step so that the chemical step for dNTP incorporation now becomes limiting (Spence *et al.*, 1995), suggesting that NNRTIs may promote nonproductive binding of dNTP substrates. Certain NNIBP residues participate in a structural element termed as the "primer grip," involved in precise positioning of the primer DNA strand in the polymerase active site (Jacobo-Molina *et al.*, 1993). NNRTI binding deforms these structural elements, thereby inhibiting reaction chemistry by preventing establishment of a catalytically competent ternary complex. Finally, the NNIBP may normally function as a hinge between the palm and thumb subdomains, and mobility of the latter

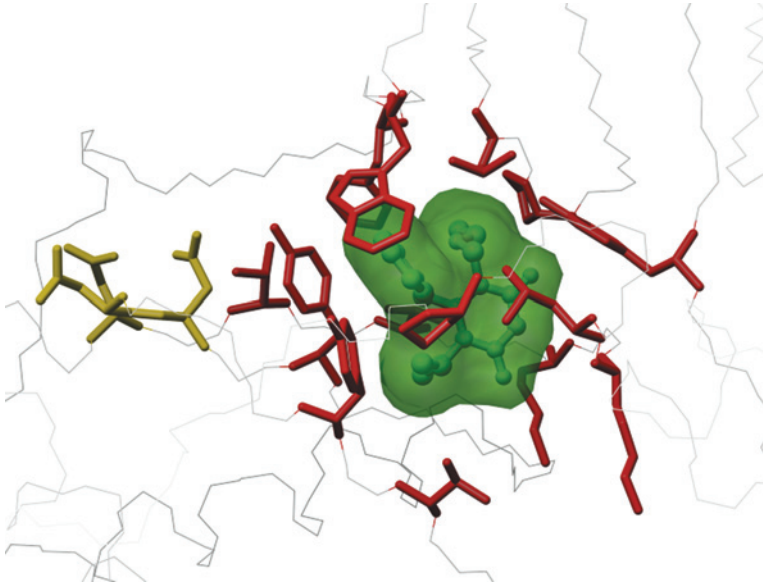


FIGURE 6 Location of the nonnucleoside inhibitor binding pocket (NNIBP) relative to the RT DNA polymerase active site. RT polymerase catalytic aspartates D110, D185, and D186 are rendered in yellow. The NNIBP is defined by the bound NNRTI (green) surrounded by amino acid residues that form the NNIBP (red). Details are provided in the text. The figure is derived from pdb file 1RT1 (Ren *et al*., 1995) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al*., 2004).

may be important for T/P translocation during DNA polymerization (Kohlstaedt *et al*., 1992; Tantillo *et al*., 1994). Binding of NNRTIs may restrict the mobility of the thumb subdomain, slowing or preventing T/P translocation and thereby inhibiting facile elongation of nascent viral DNA. This possibility is supported by numerous crystal structures of RT–NNRTI complexes (e.g., Das *et al*., 1996; Ding *et al*., 1995; Esnouf *et al*., 1995, 1997; Hopkins *et al*., 1996; Hsiou *et al*., 1998; Kohlstaedt *et al*., 1992; Ren *et al*., 1995, 2000; Smerdon *et al*., 1994), all of which show that the thumb and fingers subdomains of the RT–NNRTI complex are in an "open" conformation. This open conformation also suggests that NNRTIs may impede the formation of the "closed" ternary complex needed for incorporation of nucleotides into the growing viral DNA strand. The various mechanisms suggested are not mutually exclusive and NNRTIs may function by exerting multiple inhibitory effects on RT-catalyzed DNA synthesis.

B. NNRTI Approved for Clinical Use

1. Viramune (Nevirapine; NVP)

Nevirapine (Fig. 5, structure **12**) was the first non-NRTI to be approved (1996) for treatment of HIV-1 infection including infected children. NVP has moderate anti-HIV activity *in vitro* (EC_{50} about 100 nM). Nevirapine has excellent oral bioavailability (>90%) and a significantly prolonged serum half-life compared to NRTI. The major side effect is rash, which can be severe enough to warrant discontinuation of therapy in about 5% of patients. Nevirapine induces the CYP3A family of cytochrome P450 enzymes. The NNRTI is therefore a potent inducer of its own metabolism, leading to a twofold increase in clearance rate after about 2 weeks of administration. This induction also creates the potential for clinically significant drug interactions. Resistance to nevirapine develops rapidly both *in vitro* and *in vivo*, and resistant HIV-1 strains are detected within a month after the start of nevirapine monotherapy (Richman *et al.* 1994). Numerous mutations in the NNIBP have been associated with nevirapine resistance, including L100I, K103N, V106A, Y188C, and Y181C, although the latter appears to be the most common. Any one of these single point mutations in the NNIBP is sufficient to confer high-level HIV-1 resistance to nevirapine. Single-dose nevirapine treatment has proven useful in the prevention of maternal–fetal transmission of HIV-1 (Giaquinto *et al.*, 2006), although there are concerns about the substantial increase in NVP-resistant strains of HIV-1 that arise from such single administrations.

2. Rescriptor (Delavirdine Mesylate; DLV)

The bis(heteroaryl)piperazine (BHAP) derivative delavirdine (Fig. 5, structure **13**) was approved for treatment of HIV-1 infection in 1997. *In vitro* data have shown good inhibition properties, and the compound retains full activity against NRTI-resistant HIV-1 strains (Dueweke *et al.*, 1993a). Combinations of DLV and NRTIs provide sustained reductions in plasma viral loads as well as improvements in immunological responses (Scott and Perry, 2000). Delavirdine is generally well tolerated and is a component of recommended antiretroviral treatment regimens in combination with two NRTIs as a first-line therapy.

DLV, like NVP, undergoes hepatic metabolism by the CYP3A4 cytochrome P450 system. In contrast to nevirapine, delavirdine reduces P450 activity, thereby inhibiting its own metabolism. Delavirdine apparently also increases the steady-state concentration of the protease inhibitors saquinavir and indinavir and may therefore be useful in combination with protease inhibitors. As with NVP, resistance to delavirdine develops rapidly due to single mutations in NNIBP such as K103N, V108I, and Y181C. DLV resistance also correlates with the P236L mutation not seen in resistance to

other NNRTI. P236L does not confer NNRTI cross-resistance but may actually increase sensitivity to other NNRTIs (Dueweke *et al.*, 1993b).

3. Sustiva (Efavirenz; EFV)

Efavirenz (Fig. 5, structure **14**) is the third and to date last NNRTIs approved for clinical use in 1998. EFV is the most potent of the clinically approved NNRTI, with excellent antiviral activity against HIV-1 (EC_{50} < 1 nM) (Young *et al.*, 1995). Efavirenz has a serum half-life similar to nevirapine and crosses the blood–brain barrier, but is less orally available (≈40%). The drug has a high degree of protein binding (>99%). Efavirenz also seems to produce more pronounced side effects than NVP or DLV, including serious CNS symptoms such as insomnia, confusion, abnormal dreams, and hallucinations, which may lead to patient-initiated discontinuation of therapy. Efavirenz may also induce the CYP3A family of cytochrome P450 enzymes, thereby affecting its own metabolism and that of other drugs.

Despite these concerns, EFV has become the "mainstay" of NNRTIs, mainly because of excellent activity against many NNRTI-resistant HIV-1 strains. A preferred first-line therapy is the combination of EFV with the NRTIs AZT plus 3TC, or TDF plus FTC. The single dose combination, EFV + TDF + FTC, is marketed as Atripla (Table I). Clinically significant drug interactions have been reported with efavirenz and the protease inhibitors indinavir and saquinavir (Young *et al.*, 1995). EFV is a rather more compact molecule than NVP or DLV, but still binds to the same NNIBP pocket, with high affinity. However, interactions of EFV with NNIBP residues differ somewhat from those of NVP, and the drug can reposition in the NNIBP to accommodate the presence of certain NNRTI-resistance mutations that abrogate the activity of NVP (Ren *et al.*, 2000).

C. Investigational NNRTI

Many NNRTIs have been discovered and evaluated *in vitro* (De Clercq, 2005). However, as discussed above, only three have been approved for therapeutic use, and there have been no new NNRTIs approved for nearly a decade. Nonetheless, there are a few new NNRTIs in late stages of development.

1. Etravirine (TMC125)

The diarylpyrimidine (DAPY) derivative etravirine (Fig. 7, structure **15**) is presently in Phase III clinical trials. Etravirine has excellent potency against wild-type HIV-1 (EC_{50} = 1.4–4.8 nM) and interestingly shows some activity against HIV-2 (EC_{50} = 3.5 μM). TMC125 retains significant activity against many NNRTI-resistant variants of HIV-1, including virus with the L100I and K103N mutations that confer resistance to EFV as well as NVP and DLV (Andries *et al.*, 2004). TMC125 is a product of "rational

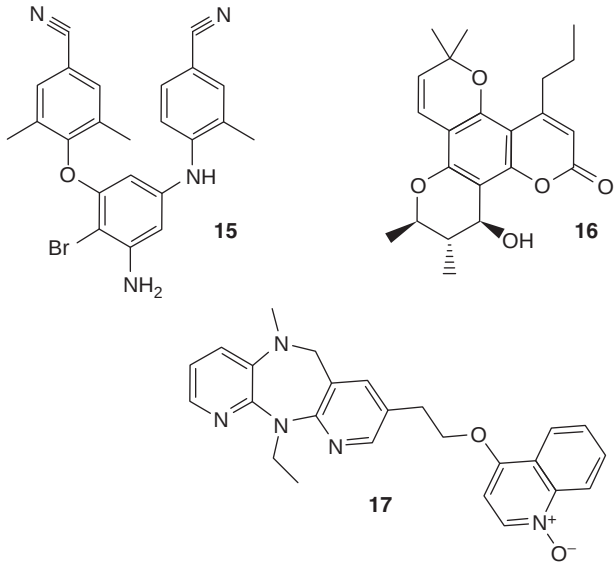


FIGURE 7 Structures of investigational NRTIs in late stage clinical trials. **15**, Etravirine (TMC125); **16**, (+)-calanolide A; **17**, BILR 355-BS. Details are in the text.

design" to develop NNRTI active against current NNRTI-resistant HIV-1. The idea was to make a very flexible molecule that could bind to the NNIBP in different conformations, enabling binding even when the NNIBP is altered by the presence of resistance mutations. Structural studies show that TMC125 has considerable torsional flexibility to allow repositioning and reorientation in the binding pocket. This flexibility may be essential for antiviral potency both against wild-type and against NNRTI-resistant HIV-1 RTs (Das *et al.*, 2004, 2005).

2. *(+)-Calanolide A*

(+)-Calanolide A (Fig. 7, structure **16**) is a dipyranocoumarin natural product originally isolated from the Malaysian tree *Caulophyllum lanigerum var. austrocoriaceum*. The compound is synthetically accessible, and resolution of the racemic (+/−)-calanolide A has shown that only the (+) enantiomer has anti-HIV activity. (+)-Calanolide A is active against a wide variety of HIV-1 strains including NRTI- and NNRTI-resistant variants (Buckheit *et al.*, 1999). (+)-Calanolide A is currently in Phase II clinical trials for use in combination therapy for HIV-1 infection. (+)-Calanolide A differs from other NNRTI in that it is partially competitive with respect to dNTP binding (Currens *et al.*, 1996b), suggesting interactions differing from those of other NNRTIs. However, like other NNRTIs, (+)-calanolide A is

active only against HIV-1 and does not inhibit HIV-2 or other retroviruses (Currens *et al*., 1996a).

3. BILR 355-BS

BILR 355-BS (Fig. 7, structure **17**) has some structural similarities to nevirapine, but shows potent antiviral activity against NVP-resistant HIV-1 strains. The extended substituent off one of the heterocyclic rings likely makes additional contacts with RT that may stabilize binding of BILR 355-BS even in the presence of NNRTI-resistance mutations. BILR 355-BS is presently in Phase II clinical trials.

D. HIV-1 Resistance to NNRTIs

HIV-1 resistance to NNRTIs correlates directly with mutations of one or more RT residues in the NNIBP (Table III). As discussed above, the NNIBP residue side chains may adapt to each NNRTI in a specific manner; thus, mutations of some NNIBP residue side chains may confer resistance to one class of NNRTI, while not affecting potency of another (Balzarini *et al*., 1996). Nevertheless, mutations of certain residues such as K103 can lead to broad-spectrum NNRTI resistance, possibly since these specific residues form critical contacts with the NNRTI pharmacophore.

NNRTI resistance may arise in a number of ways. Mutations of specific residues may remove favorable interactions between the inhibitor and the NNIBP. For example, the Y181C/I and Y188C/L mutations would eliminate π-stacking interactions with the aromatic ring systems of the NNRTI. Other mutations may introduce steric barriers to NNRTI binding. For example, the G190A/E/Q/T mutations all introduce bulky side chains, which may prevent binding of NNRTI by sterically interfering with NNRTI functional groups such as the cyclopropyl ring of nevirapine. Mutations may introduce or eliminate inter-residue contacts in the NNIBP that could interfere with the ability of other NNIBP side chains to fold down over the NNRTI to maintain stable binding. It has also been suggested that mutations in the NNIBP may not affect the rate of binding of NNRTIs such as nevirapine, but

TABLE III Clinically Significant Mutations in Reverse Transcriptase Associated with HIV Resistance to NNRTI

Drug	*Mutations*	*Resistance phenotype*
Nevirapine	L100I, K103N, V106A, V108I, Y181C, Y188C/L/H, G190S/A	Reduced affinity
Delavirdine	K103N, V106M, Y181C, Y188L, P236L	Reduced affinity
Efavirenz	L100I, K103N, V106M, V108I, Y181C/I, Y188L, G190S/A, P225H	Reduced affinity

rather may lead to increases in the rate of inhibitor dissociation (Spence *et al.*, 1996). Any or all of these possibilities would diminish the overall affinity of RT for NNRTIs with attendant reduction in inhibitory potency.

1. Mutations Associated with HIV-1 Resistance to NNRTIs

Table III lists most of the clinically seen NNRTI-resistance mutations and the resistance phenotype associated with these. High-level resistance to more potent NNRTIs such as efavirenz generally requires multiple mutations in the NNIBP. Resistance to NNRTIs usually emerges rapidly when administered as monotherapy or incomplete virus suppression (Conway *et al.*, 2001). Some NNRTI resistance mutations may also compromise viral replication capacity even in the absence of NNRTIs, possibly by altering the conformation of the dNTP binding pocket (Van Vaerenbergh *et al.*, 2000) and/or by changes in RT RNaseH activity (Archer *et al.*, 2000). The first RT mutations correlated with NNRTI resistance were K103N and Y181C (Mellors *et al.*, 1992; Richman *et al.*, 1991), mutations that are associated with resistance to each of the clinically used NNRTIs. Other relatively common mutations include L100I and V106A, whereas the P236L mutation is unique in that it arises only in resistance to delavirdine (Dueweke *et al.*, 1993b).

a. Mutations in Residues 98–108

i. L100I provides low-level resistance to NVP and intermediate resistance to DLV and EFV (Byrnes *et al.*, 1993; Young *et al.*, 1995). This mutation usually occurs along with K103N in patients treated with EFV, and the L100V + K103N mutation combination provides high-level resistance to all three approved NNRTIs (Bacheler *et al.*, 2000). Interestingly, the L100I mutation partially reverses resistance to AZT and TDF arising from the T215Y NRTI-resistance mutation (Larder and Stammers, 1999).

ii. K103N is the most common NNRTI resistance mutation seen in patients on NNRTI therapy (Conway *et al.*, 2001; Deeks, 2001). This mutation alone causes a 20- to 50-fold resistance to each of the clinically used NNRTIs, and K103N alone is sufficient to cause virologic failure with each of the NNRTIs. The mutation K103S occurs in about 1% of NNRTI-treated persons and causes about 10-fold resistance to efavirenz and delavirdine, and 30-fold resistance to nevirapine (Harrigan *et al.*, 2003). Residue K103 is on the outer rim of the NNIBP in the vicinity of the entrance to the pocket. Crystal structures of K103N RT, both unliganded and in complex with NNRTIs, show that the K103N mutation has little impact on RT structure, but in the unliganded RT this mutation forms additional hydrogen bonds not found in the wild-type enzyme. These additional bonds stabilize the closed pocket form of the enzyme and thus interfere with inhibitor binding (Hsiou *et al.*, 2001).

iii. V106A leads to greater than 30-fold resistance to NVP but only 2- to 5-fold resistance to DLV and EFV (Byrnes *et al.*, 1993). V106M occurs

in subtype C isolates in patients failing NNRTI therapy, but is rarely seen in subtype B virus (Brenner *et al.*, 2003).

b. Mutations in Residues 179–190

i. Y181C is commonly seen in resistance to NVP and provides more than 30-fold resistance to NVP and DLV, but only minor resistance to EFV (Byrnes *et al.*, 1993; Young *et al.*, 1995). Introduction of the Y181C mutation removes hydrophobic and π-stacking interactions important for binding of NNRTIs.

ii. Y188L is much less common than Y181C, but leads to high-level resistance to each of the three clinically used NNRTIs (Byrnes *et al.*, 1993; Young *et al.*, 1995). As with Y181C, the Y188L mutation eliminates hydrophobic and π-stacking interactions important for binding of many NNRTIs. The Y188H substitution occurs infrequently and provides 30-fold resistance to (+)-calanolide A, but surprisingly does not confer resistance to NVP (Quan *et al.*, 1999).

iii. G190A leads to high-level resistance to nevirapine and lesser resistance to EFV, whereas G190S causes high-level resistance to both NVP and EFV. Interestingly, HIV-1 with G190A/S mutations in RT are hypersusceptible to delavirdine (Huang *et al.*, 2003).

c. Other NNRTI-Resistance Mutations Mutations in other regions of the NNIBP occur less frequently than those occurring in residues 98–108 and 179–190. P225H occurs with K103N in patients receiving efavirenz. The combination of two substitutions K103N and P225H causes about 100-fold resistance to EFV and NVP, but only 10-fold resistance to DLV because P225H confers hypersusceptibility to delavirdine (Pelemans *et al.*, 1998). Y318F confers high-level resistance to DLV but only minor resistance to NVP and EFV (Harrigan *et al.*, 2002). Residue E138 of the p51 subunit contributes to the NNIBP. Mutations such as E138K confer resistance to experimental NNRTIs such as TSAO, but do not affect sensitivity to clinically used NNRTIs (Balzarini *et al.*, 1994).

E. Interactions Between NNRTI and NRTI Resistance Mutations

NNRTIs and NRTIs bind to different sites on RT and thus combinations of these two classes of drugs provide additivity or even synergy in inhibition and antiviral potency. NRTI + NNRTI combinations are a standard first-line therapy option for treatment of HIV-1 infection. While drug combinations will lead to virus with both NRTI- and NNRTI-resistance mutations, such mutations can interact in unpredictable but potentially beneficial ways. For example, the NNRTI-resistant mutations, Y181C and L100I, hypersensitize both wild-type and TAM-mutant HIV-1 to AZT (Byrnes *et al.*, 1993)

as well as to the nucleotide RT inhibitor TDF (Parkin *et al*., 2003). This hypersensitivity may be due to mutation-induced diminution of the rate of NRTI excision (Selmi *et al*., 2003). As a corollary, HIV-1 isolates containing multiple NRTI resistance mutations appear to be more susceptible to the currently approved NNRTIs than are isolates lacking NRTI resistance mutations (Whitcomb *et al*., 2002). The NRTI resistance mutations can lead to up to fivefold increases in NNRTI sensitivity in HIV-1 also containing NNRTI-resistance mutations (Shulman *et al*., 2001). The molecular mechanisms for these resensitization phenomena are not presently clear.

F. Use of NNRTI as Microbicides

Heterosexual contact is the primary mode of transmission of HIV infection worldwide. Vaginal microbicides that inhibit sexual transmission of HIV could minimize the continued spread of HIV. Microbicidal agents should act directly on the virus without the need for prior metabolic activa-

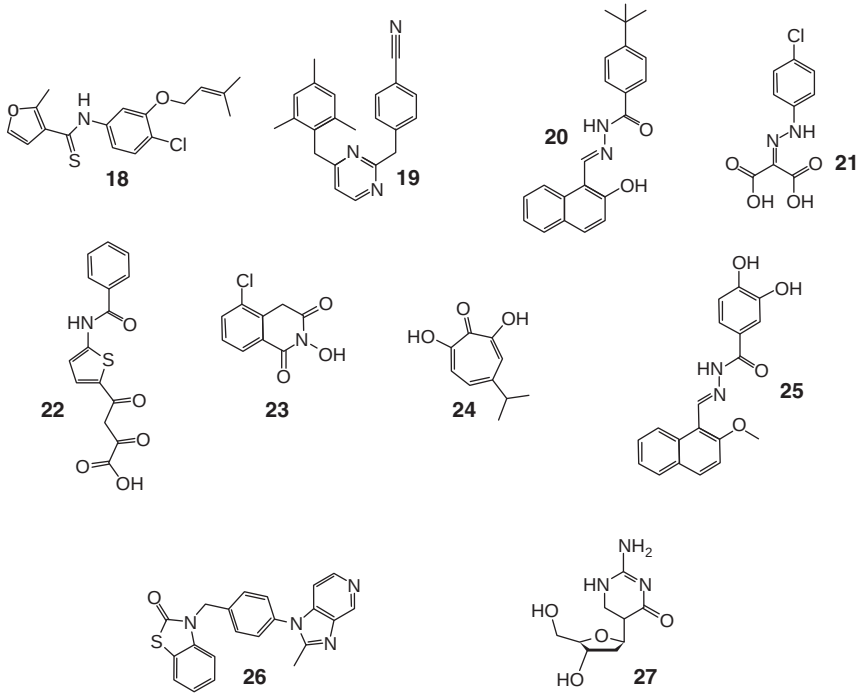


FIGURE 8 Structures of investigational RT inhibitors directed at novel HIV-1 RT targets or for novel uses. **18**, UC781; **19**, TMC120; **20**, BBNH; **21**, CPHM; **22**, Merck diketoacid analog RNH inhibitor; **23**, *N*-hydroxyimide RNH inhibitor; **24**, hydroxyl tropolone RNH inhibitor; **25**, DHBNH; **26**, CP-94,707; **27**, KP-1212. Details are in the text.

tion, and should act at replication steps prior to proviral DNA integration. In addition, the ideal retrovirucidal agent should be absorbable by uninfected cells in order to provide a barrier to infection by residual active virus and HIV-infected cells in sexual fluids, and it should be effective at nontoxic concentrations readily attainable by topical application *in vivo*. NNRTIs fulfill many of these parameters and two experimental NNRTIs, UC781 (Fig. 8, structure **18**), and TMC-120 (Fig. 8, structure **19**) are in Phase I clinical trials as potential microbicides. Most of the published data available relate to UC781.

Biochemical and virological studies (Barnard *et al.*, 1997; Borkow *et al.*, 1997a; Hossain and Parniak, 2006) combined with preliminary preclinical evaluations (Balzarini *et al.*, 1998; Patton *et al.*, 2007) show that UC781 has considerable potential as an anti-HIV microbicide. HIV-1 infectivity is eliminated in a concentration-dependent manner following transient exposure of isolated virions to low concentrations of UC781 ($<$50 nM). When chronically infected H9 lymphocytoid cells or peripheral blood lymphocytes isolated from HIV-1-infected individuals are transiently exposed to UC781, HIV-1 subsequently produced in the absence of the drug is noninfectious. Importantly, transient exposure of uninfected cells to UC781 renders these cells refractory to subsequent HIV-1 infection; this "chemical barrier" or "memory effect" persists for extended periods following a single low-dose UC781 treatment. The establishment of this barrier suggests that while UC781 is taken up rapidly by cells, it enters some cellular compartment, accessible to HIV-1, in which it may remain for extended periods to exert a continuing protective effect. Recent data show that UC781 has microbicidal activity even against NNRTI-resistant strains of HIV-1, including those with the K100I + K103N mutations in RT associated with high-level resistance to EFV (Hossain and Parniak, 2006). Finally, preclinical animal studies in macaques have shown UC781 to be safe even with repeated topical vaginal application (Patton *et al.*, 2007).

Interestingly, not all NNRTIs are useful as microbicides. Transient exposure of HIV-1 virions to nevirapine or DLV did not affect viral infectivity. Similarly, no "chemical barrier" to HIV infection was established by transient treatment of uninfected cells with either NVP or DLV; these cells were as readily infected by HIV-1 as untreated cells (Borkow *et al.*, 1997a; Motakis and Parniak, 2002).

VI. Other Inhibitors of HIV-1 RT

All clinically used RT inhibitors (Table I) target the DNA polymerase activity of this enzyme. However, HIV-1 RT provides several additional potential targets for the discovery and development of novel therapeutics.

A. Inhibitors of HIV-1 RT RNH

Retroviruses carry their genomic information as (+)RNA but must replicate through a double-strand DNA intermediate. The conversion of retroviral single-strand genomic (+)RNA to double-strand DNA (dsDNA) generates an RNA/DNA heteroduplex intermediate, the RNA component of which must be removed so that the nascent (−)DNA can be copied to complete viral dsDNA synthesis. The removal of the RNA in the RNA/DNA heteroduplex is carried out by the RT-associated enzyme activity, RNH. The RT DNA polymerase and RNH active sites are spatially distinct, with RNH activity localized in the C-terminal 15-kDa subdomain of the 66-kDa subunit of the HIV-1 RT 66/51 heterodimer (Fig. 1). RT RNH activity is essential for HIV-1 replication at several steps of reverse transcription. RNH is involved in degrading viral genomic (+)RNA to allow (−)DNA strand transfer and (+)DNA strand synthesis (Peliska and Benkovic, 1992). RNH is also important for the generation of polypurine tract primers that are used for the initiation of (+)DNA strand synthesis as well as for the removal of these primers (Cirino *et al.*, 1995). Cellular RNH cannot function in HIV replication, since HIV-1 virions with inactive RNH are unable to replicate (Tisdale *et al.*, 1991). HIV-1 RT RNH is thus an interesting target for antiretroviral drug discovery (Tramontano, 2006).

Despite the attractiveness of HIV RT RNH as a potential target, very few inhibitors of this enzyme activity have been discovered and only six RNH inhibitors with reasonable *in vitro* potencies have been described (Fig. 8, structures **20–25**). Unfortunately, most of these are of no therapeutic value due to limited cell uptake, cytotoxicity, etc. We were the first to describe an RNH inhibitor with reasonable potency (Borkow *et al.*, 1997b). The acylhydrazone *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH; Fig. 8, structure **20**) inhibits HIV-1 RT RNH ($IC_{50} \approx 3\ \mu M$) as well as Moloney murine leukemia virus RT RNH and *Escherichia coli* RNH. While BBNH has antiviral activity, it is too toxic for consideration as a possible therapeutic. The aryl hydrazone 4-chlorophenyl hydrazone mesoxalate (CPHM; Fig. 8, structure **21**) inhibits RT RNH with potency similar to that of BBNH (Davis *et al.*, 2000). While BBNH and CPHM share some structural similarity, they show significant differences in inhibitory mechanism. BBNH is in fact a bifunctional inhibitor of RT, inhibiting both the DNA polymerase and RNH activities of the enzyme (Borkow *et al.*, 1997b). In contrast, CPHM is specific for RNH and does not inhibit RT DNA polymerase activity. CPHM has no antiviral activity, possibly due to difficulty in cell uptake of this charged molecule. The Merck group described a diketo acid RNH inhibitor, 4-[5-benzoylamino) thien-2-yl]-2,4-dioxobutanoic acid (Fig. 8, structure **22**) that was discovered as an offshoot of their integrase inhibitor discovery program (Shaw-Reid *et al.*, 2003). Like CPHM, this molecule does not show antiviral activity. Two new

classes of RNH inhibitors have recently been described, the *N*-hydroxyimides (Hang *et al.*, 2004; Klumpp *et al.*, 2003) (Fig. 8, structure **23**) and hydroxy tropolones (Budihas *et al.*, 2005) (Fig. 8, structure **24**). Neither of these compounds are therapeutic candidates since they show significant cytotoxicity.

A crystal structure of HIV-1 RT complexed with an acylhydrazone analog of BBNH, dihydroxy benzoyl naphthyl hydrazone (DHBNH), has recently been described (Himmel *et al.*, 2006). Unlike BBNH, DHBNH (Fig. 8, structure **25**) is a specific inhibitor of RT RNH activity ($IC_{50} \approx 0.5\ \mu M$) and does not inhibit RT DNA polymerase activity. DHBNH is the first RNH inhibitor to show reasonable antiviral potency in the absence of cytotoxicity and is effective against a variety of drug-resistant HIV-1 RT mutants (Himmel *et al.*, 2006). Interestingly, DHBNH binds more than 50 Å away from the RNH active site, at a novel site near both the polymerase active site and the NNIBP RTIs binding pocket (Fig. 9). The mechanism by which such binding can inhibit RT RNH is not clear, but may involve repositioning of the polymerase primer grip and the thumb of RT in a

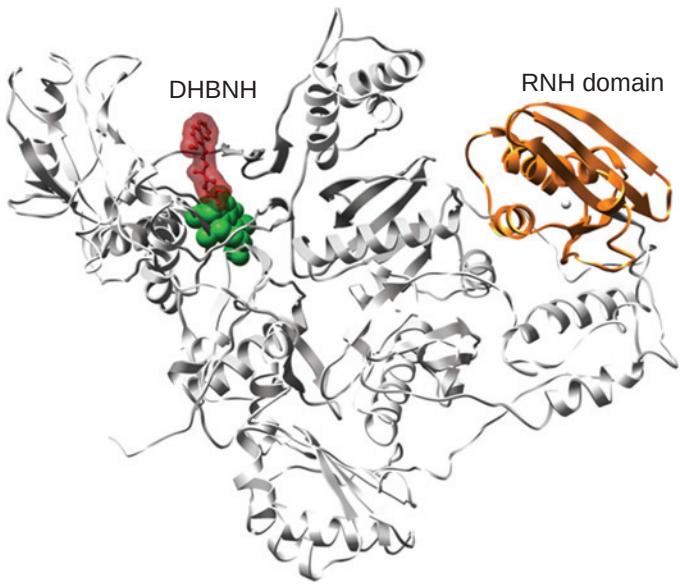


FIGURE 9 Location of the ribonuclease H (RNH)-specific inhibitor dihydroxy benzoyl naphthyl hydrazone (DHBNH) relative to the RNH domain of HIV-1 RT. DHBNH is rendered in red spacefill. The nonnucleoside inhibitor binding pocket (NNIBP) is indicated by residues in green spacefill. The figure is derived from pdb file 215J (Himmel *et al.*, 2006) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004).

manner that alters the trajectory of the nucleic acid through the RNH, thereby preventing RNH-catalyzed hydrolysis of the RNA strand (Himmel *et al.*, 2006). Alternatively, DHBNH and other acylhydrazones may bind to a second site in or near the RNH domain that was not seen in the crystal. Additional studies are needed to validate this possibility.

Selective HIV RNH inhibitors are likely to provide significant clinical benefit in combination therapies, as they are likely to be fully active against all current drug-resistant variants of HIV (Klumpp and Mirzadegan, 2006). The recent development of a high throughput screening assay to identify inhibitors of HIV RNH (Parniak *et al.*, 2003) should facilitate the discovery process for the identification of new RNH inhibitors.

B. Inhibitors of HIV-1 RT Dimerization

Although the gene for HIV-1 RT encodes a 66-kDa protein, the biologically functional form of RT is an obligate p66/p51 heterodimer. Inhibition of RT dimerization during HIV-1 assembly and maturation, or disruption of mature RT dimer stability, might be novel targets for new RT inhibitor development. Several subunit interface peptides of 15–19 amino acids in length comprising sequences from the RT connection subdomain inhibited the reassociation of organic solvent denatured RT subunits and thus activity of RT (Divita *et al.*, 1995). Peptide therapeutics are not ideal due to limited bioavailability and cell uptake as well as the possibility of immune recognition, all of which would limit accessibility of the peptide to its target.

A number of small molecules have been found to disrupt HIV-1 RT p66/p51 heterodimer stability. These "molecular crowbars" may be useful leads in the development of RT inhibitors that function at multiple stages of HIV-1 replication. The molecule 1-[2′,5′-bis-*O*-(*t*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-3-ethylthymine ($TSAOe^3T$) is a highly modified nucleoside that acts in a manner similar to NNRTIs (Balzarini *et al.*, 1992a,b). Interaction of $TSAOe^3T$ with RT results in a 4 kcal mol^{-1} decrease in the strength of the p66–p51 subunit association (Camarasa *et al.*, 2006a,b; Sluis-Cremer *et al.*, 2000a), a substantial destabilization of RT subunit interactions. NNRTIs such as nevirapine and UC781 do not alter the subunit association strength. Certain acylhydrazones such as BBNH destabilize HIV-1 RT p66–p51 subunit interactions, similar to TSAO, and inhibit both RT DNA polymerase and RNH activities (Sluis-Cremer *et al.*, 2002).

C. Inhibitors of the Initiation of Reverse Transcription

Reverse transcription initiates from a tRNA primer annealed to a specific primer binding sequence in the HIV genome (Gotte *et al.*, 1999). Initiation is rate limiting in reverse transcription and has been proposed as a potential drug target unique from RT-catalyzed DNA synthesis that is

targeted by current NRTIs and NNRTIs (Pata *et al.*, 2004; Rigourd *et al.*, 2002). The compound CP-94,707 (Fig. 8, structure **26**) specifically inhibits initiation by binding to a novel site on RT distinct from the NNIBP (Pata *et al.*, 2004). We have recently found that the "RNH-specific" acylhydrazone DHBNH (Fig. 8, structure **25**), which does not inhibit RT-catalyzed processive DNA synthesis, does in fact inhibit the initiation of reverse transcription from an RNA primer, and this inhibition of initiation may be a major factor in the antiviral activity of this compound (Himmel *et al.*, 2006).

D. RT-Directed Mutagenic Inducers

The compound 5,6-dihydro-5-aza-2′-deoxycytidine (KP-1212; Fig. 8, structure **27**) is a non-chain-terminating NRTI deoxycytidine nucleoside analog that is randomly incorporated into HIV DNA by RT (Harris *et al.*, 2005). The molecule comprises a normal deoxyribose sugar and a modified nonplanar heterocyclic base that tautomerizes between a cytosine form and a thymine form and thus base pairs to multiple nucleotides in DNA. Unlike other NRTIs, KP-1212 does not block viral DNA synthesis, but rather gets incorporated throughout the viral DNA thereby increasing the mutation rate of HIV by mispairing. This results in defective viruses leading to lethal mutagenesis (Loeb *et al.*, 1999; Smith *et al.*, 2005). No cross-resistance with HIV strains resistant to other NRTIs has yet been found (Murakami *et al.*, 2005).

VII. Conclusion

Despite the increasing prevalence of NRTI- and NNRTI-resistant strains of HIV-1, RT remains an essential drug target for the treatment of HIV infection. As described in this chapter, there are a number of new NRTIs and NNRTIs, with improved resistance profiles, in late stage clinical development, and there are many others in preclinical development. In addition to DNA polymerase activity, RT also presents a variety of additional targets for antiretroviral drug discovery, including RNH, RT subunit dimerization, and initiation of reverse transcription, and small molecule leads have been described for each of these targets. HIV-1 RT will continue to be an important therapeutic target for many years to come.

Acknowledgments

Research in the Parniak laboratory is supported by grants from the National Institutes of Health (AI51661, AI52010, AI60452, and GM66671). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

References

Andries, K., Azijn, H., Thielemans, T., Ludovici, D., Kukla, M., Heeres, J., Janssen, P., De Corte, B., Vingerhoets, J., Pauwels, R., and de Bethune, M. P. (2004). TMC125, a novel next-generation nonnucleoside reverse transcriptase inhibitor active against nonnucleoside reverse transcriptase inhibitor-resistant human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* **48**, 4680–4686.

Archer, R. H., Dykes, C., Gerondelis, P., Lloyd, A., Fay, P., Reichman, R. C., Bambara, R. A., and Demeter, L. M. (2000). Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. *J. Virol.* **74**, 8390–8401.

Arion, D., Borkow, G., Gu, Z., Wainberg, M. A., and Parniak, M. A. (1996). The K65R mutation confers increased DNA polymerase processivity to HIV-1 reverse transcriptase. *J. Biol. Chem.* **271**, 19860–19864.

Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998). Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): Increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* **37**, 15908–15917.

Bacheler, L. T., Anton, E. D., Kudish, P., Baker, D., Bunville, J., Krakowski, K., Bolling, L., Aujay, M., Wang, X. V., Ellis, D., Becker, M. F., Lasut, A. L., *et al.* (2000). Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. *Antimicrob. Agents Chemother.* **44**, 2475–2484.

Balzarini, J., Perez-Perez, M. J., San-Felix, A., Schols, D., Perno, C. F., Vandamme, A. M., Camarasa, M. J., and De Clercq, E. (1992a). 2′,5′-Bis-O-(tert-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine (TSAO) nucleoside analogues: Highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **89**, 4392–4396.

Balzarini, J., Perez-Perez, M. J., San-Felix, A., Camarasa, M. J., Bathurst, I. C., Barr, P. J., and De Clercq, E. (1992b). Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2′,5′-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) thymine (TSAO-T). *J. Biol. Chem.* **267**, 11831–11838.

Balzarini, J., Holy, A., Jindrich, J., Naesens, L., Snoeck, R., Schols, D., and De Clercq, E. (1993). Differential antiherpes virus and antiretrovirus effects of the (S) and (R) enantiomers of acyclic nucleoside phosphonates: Potent and selective *in vitro* and *in vivo* antiretrovirus activities of (R)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. *Antimicrob. Agents Chemother.* **37**, 332–338.

Balzarini, J., Karlsson, A., Sardana, V. V., Emini, E. A., Camarasa, M. J., and De Clercq, E. (1994). Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K)RT HIV-1

mutants or select for highly resistant (Y181C→C181I) RT HIV-1 mutants. *Proc. Natl. Acad. Sci. USA* **91**, 6599–6603.

Balzarini, J., Pelemans, H., Aquaro, S., Perno, C. F., Witvrouw, M., Schols, D., De Clercq, E., and Karlsson, A. (1996). Highly favorable antiviral activity and resistance profile of the novel thiocarboxanilide pentenyloxy ether derivatives UC-781 and UC-82 as inhibitors of human immunodeficiency virus type 1 replication. *Mol. Pharmacol.* **50**, 394–401.

Balzarini, J., Naesens, L., Verbeken, E., Laga, M., Van Damme, L., Parniak, M. A., Van Melaert, L., Anne, J., and De Clercq, E. (1998). Preclinical studies on thiocarboxanilide UC781 as a virucidal agent. *AIDS* **12**, 1129–1138.

Bang, L. M., and Scott, L. J. (2003). Emtricitabine: An antiretroviral agent for HIV infection. *Drugs* **63**, 2413–2424.

Barnard, J., Borkow, G., and Parniak, M. A. (1997). The thiocarboxanilide UC781 is a tight-binding nonnucleoside inhibitor of HIV-1 reverse transcriptase. *Biochemistry* **36**, 7786–7792.

Borkow, G., Barnard, J., Nguyen, T. M., Belmonte, A., Wainberg, M. A., and Parniak, M. A. (1997a). Chemical barriers to HIV-1 infection: Retrovirucidal activity of UC781, a thiocarboxanilide nonnucleoside inhibitor of HIV-1 reverse transcriptase. *J. Virol.* **71**, 3023–3030.

Borkow, G., Fletcher, R. S., Barnard, J., Arion, D., Motakis, D., Dmitrienko, G. I., and Parniak, M. A. (1997b). Inhibition of the ribonuclease H and DNA polymerase activities of HIV-1 reverse transcriptase by N-(4-tert-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. *Biochemistry* **36**, 3179–3185.

Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2002a). Nucleoside analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. *J. Virol.* **76**, 9143–9151.

Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2002b). The M184V mutation reduces the selective excision of zidovudine 5,-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. *J. Virol.* **76**, 3248–3256.

Brenner, B., Turner, D., Oliveira, M., Moisi, D., Detorio, M., Carobene, M., Marlink, R. G., Schapiro, J., Roger, M., and Wainberg, M. A. (2003). A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS* **17**, F1–F5.

Buckheit, R. W., Jr, White, E. L., Fliakas-Boltz, V., Russell, J., Stup, T. L., Kinjerski, T. L., Osterling, M. C., Weigand, A., and Bader, J. P. (1999). Unique anti-human immunodeficiency virus activities of the nonnucleoside reverse transcriptase inhibitors calanolide A, costatolide and dihydrocostatolide. *Antimicrob. Agents Chemother.* **43**, 1827–1834.

Budihas, S. R., Gorshkova, I., Gaidamakov, S., Wamiru, A., Bona, M. K., Parniak, M. A., Crouch, R. J., McMahon, J. B., Beutler, J. A., and Le Grice, S. F. (2005). Selective inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones. *Nucleic Acids Res.* **33**, 1249–1256.

Byrnes, V. W., Sardana, V. V., Schleif, W. A., Condra, J. H., Waterbury, J. A., Wolfgang, J. A., Long, W. J., Schneider, C. L., Schlabach, A. J., and Wolanski, B. S. (1993). Comprehensive mutant enzyme and viral variant assessment of human immunodeficiency virus type 1 reverse transcriptase resistance to nonnucleoside inhibitors. *Antimicrob. Agents Chemother.* **37**, 1576–1579.

Caliendo, A. M., Savara, A., An, D., DeVore, K., Kaplan, J. C., and D'Aquila, R. T. (1996). Effects of zidovudine-selected human immunodeficiency virus type 1 reverse transcriptase amino acid substitutions on processive DNA synthesis and viral replication. *J. Virol.* **70**, 2146–2153.

Camarasa, M. J., Velazquez, S., San-Felix, A., Perez-Perez, M. J., and Gago, F. (2006a). Dimerization inhibitors of HIV-1 reverse transcriptase, protease and integrase: A single mode of inhibition for the three HIV enzymes? *Antiviral Res.* **71**, 260–267.

Camarasa, M. J., Velazquez, S., San-Felix, A., Perez-Perez, M. J., Bonache, M. C., and De Castro, S. (2006b). TSAO derivatives, inhibitors of HIV-1 reverse transcriptase dimerization: Recent progress. *Curr. Pharm.* **12**, 1895–1907.

Cirino, N. M., Cameron, C. E., Smith, J. S., Rausch, J. W., Roth, M. J., Benkovic, S. J., and Le Grice, S. F. J. (1995). Divalent cation modulation of the ribonuclease H functions of human immunodeficiency virus reverse transcriptase. *Biochemistry* **34**, 9936–9943.

Coates, J. A., Cammack, N., Jenkinson, H. J., Jowett, A. J., Jowett, M. I., Pearson, B. A., Penn, C. R., Rouse, P. L., Viner, K. C., and Cameron, J. M. (1992). (-)-2′-deoxy-3′-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication *in vitro*. *Antimicrob. Agents Chemother.* **36**, 733–739.

Conway, B., Wainberg, M. A., Hall, D., Harris, M., Reiss, P., Cooper, D., Vella, S., Curry, R., Robinson, P., Lange, J. M., and Montaner, J. S. (2001). Development of drug resistance in patients receiving combinations of zidovudine, didanosine and nevirapine. *AIDS* **15**, 1269–1274.

Currens, M. J., Gulakowski, R. J., Mariner, J. M., Moran, R. A., Buckheit, R. W., Jr., Gustafson, K. R., McMahon, J. B., and Boyd, M. R. (1996a). Antiviral activity and mechanism of action of calanolide A against the human immunodeficiency virus type-1. *J. Pharmacol. Exp. Ther.* **279**, 645–651.

Currens, M. J., Mariner, J. M., McMahon, J. B., Boyd, M. R., Shulman, N., and Winters, M. (1996b). Kinetic analysis of inhibition of human immunodeficiency virus type-1 reverse transcriptase by calanolide A. *J. Pharmacol. Exp. Ther.* **279**, 652–661.

Dahlberg, J. E., Mitsuya, H., Blam, S. B., Broder, S., and Aaronson, S. A. (1987). Broad spectrum antiretroviral activity of 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **84**, 2469–2473.

Daluge, S. M., Good, S. S., Faletto, M. B., Miller, W. H., St Clair, M. H., Boone, L. R., Tisdale, M., Parry, N. R., Reardon, J. E., Dornsife, R. E., Averett, D. R., and Krenitsky, T. A. (1997). 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. *Antimicrob. Agents Chemother.* **41**, 1082–1093.

Das, K., Ding, J., Hsiou, Y., Clark, A. D., Jr., Moereels, H., Koymans, L., Andries, K., Pauwels, R., Janssen, P. A. J., Boyer, P. L., Clark, P., Smith, R. H., Jr., *et al.* (1996). Crystal structures of 8-Cl and 9-Cl TIBO complexed with wild-type RT and 8-Cl TIBO complexed with the Tyr181Cys HIV-1 RT drug-resistant mutant. *J. Mol. Biol.* **264**, 1085–1100.

Das, K., Clark, A. D., Jr., Lewi, P. J., Heeres, J., De Jonge, M. R., Koymans, L. M., Vinkers, H. M., Daeyaert, F., Ludovici, D. W., Kukla, M. J., De Corte, B., Kavash, R. W., *et al.* (2004). Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J. Med. Chem.* **47**, 2550–2560.

Das, K., Lewi, P. J., Hughes, S. H., and Arnold, E. (2005). Crystallography and the design of anti-AIDS drugs: Conformational flexibility and positional adaptability are important in the design of non-nucleoside HIV-1 reverse transcriptase inhibitors. *Prog. Biophys. Mol. Biol.* **88**, 209–231.

Davis, W. R., Tomsho, J., Nikam, S., Cook, E. M., Somand, D., and Peliska, J. A. (2000). Inhibition of HIV-1 reverse transcriptase-catalyzed DNA strand transfer reactions by 4-chlorophenylhydrazone of mesoxalic acid. *Biochemistry* **39**, 14279–14291.

De Clercq, E. (1998). The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. *Antiviral Res.* **38**, 153–179.

De Clercq, E. (2005). New approaches toward anti-HIV chemotherapy. *J. Med. Chem.* **48**, 297–1313.

Deeks, S. G. (2001). Nonnucleoside reverse transcriptase inhibitor resistance. *J. Acquir. Immune Defic. Syndr.* **26**(Suppl. 1), S25–S33.

De Muys, J., Gourdeau, H., Nguyen-Ba, N., Taylor, D., Ahmed, P., and Mansour, T. (1999). Anti-human immunodeficiency virus type 1 activity, intracellular metabolism, and pharmacokinetic evaluation of 2′-deoxy-3′-oxa-4′-thiocytidine. *Antimicrob. Agents Chemother.* **43**, 1835–1844.

Deval, J., Selmi, B., Boretto, J., Egloff, M. P., Guerreiro, C., Sarfati, S., and Canard, B. (2002). The molecular mechanism of multidrug resistance by the Q151M human immunodeficiency virus type 1 reverse transcriptase and its suppression using alpha-boranophosphate nucleotide analogues. *J. Biol. Chem.* **277**, 42097–42104.

Dharmasena, S., Poncracz, Z., Arnold, E., Sarafianos, S. G., and Parniak, M. A. (2007). 3′-azido-3′-deoxythymidine(5′)tetraphospho(5′)-adenosine, the product of ATP-dependent excision of chain-terminating 3′-azido-3′-deoxythymidine-5-monophosphate, is a potent chain-terminating substrate for HIV-1 reverse transcriptase. *Biochemistry* **46**, 828–836.

Diallo, K., Marchand, B., Wei, X., Cellai, L., Gotte, M., and Wainberg, M. A. (2003). Diminished RNA primer usage associated with the L74V and M184V mutations in the reverse transcriptase of human immunodeficiency virus type 1 provides a possible mechanism for diminished viral replication capacity. *J. Virol.* **77**, 8621–8632.

Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P. A., Hughes, S. H., and Arnold, E. (1995). Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat. Struct. Biol.* **2**, 407–415.

Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, J. A. D., Jacobo-Molina, A., Tantillo, C., Hughes, S. H., and Arnold, E. (1998). Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with double-stranded DNA and an antibody Fab fragment at 2.8 Å resolution. *J. Mol. Biol.* **284**, 1095–1111.

Divita, G., Rittinger, K., Restle, T., Immendorfer, U., and Goody, R. S. (1995). Conformational stability of dimeric HIV-1 and HIV-2 reverse transcriptases. *Biochemistry* **34**, 16337–16346.

Domingo, E., Menendez-Arias, L., Quinones-Mateu, M. E., Holguin, A., Gutierrez-Rivas, M., Martinez, M. A., Quer, J., Novella, I. S., and Holland, J. J. (1997). Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. *Prog. Drug Res.* **48**, 99–128.

Dueweke, T. J., Poppe, S. M., Romero, D. L., Swaney, S. M., So, A. G., Downey, K. M., Althaus, I. W., Reusser, F., Busso, M., and Resnick, L. (1993a). U-90152, a potent inhibitor of human immunodeficiency virus type 1 replication. *Antimicrob. Agents Chemother.* **37**, 1127–1131.

Dueweke, T. J., Pushkarskaya, T., Poppe, S. M., Swaney, S. M., Zhao, J. Q., Chen, I. S. Y., Stevenson, M., and Tarpley, W. G. (1993b). A mutation in reverse transcriptase of bis (heteroaryl) piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors. *Proc. Natl. Acad. Sci. USA* **90**, 4713–4717.

Dutschman, G., Bridges, E., Liu, S., Gullen, E., Guo, X., Kukhanova, M., and Cheng, Y. C. (1998). Metabolism of 2′,3′-dideoxy-2′,3′-didehydro-beta-L(–)-5-fluorocytidine and its activity in combination with clinically approved anti-human immunodeficiency virus beta-D(+) nucleoside analogs *in vitro*. *Antimicrob. Agents Chemother.* **42**, 1799–1804.

Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., and Stuart, D. (1995). Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat. Struct. Biol.* **2**, 303–308.

Esnouf, R. M., Ren, J., Hopkins, A. L., Ross, C. K., Jones, E. Y., Stammers, D. K., and Stuart, D. I. (1997). Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proc. Natl. Acad. Sci. USA* **94**, 3984–3989.

Faletto, M. B., Miller, W. H., Garvey, E. P., St Clair, M. H., Daluge, S. M., and Good, S. S. (1997). Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. *Antimicrob. Agents Chemother.* **41**, 1099–1107.

Fitzgibbon, J. E., Howell, R. M., Haberzettl, C. A., Sperber, S. J., Gocke, D. J., and Dubin, D. T. (1992). Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2′,3′-dideoxycytidine. *Antimicrob. Agents Chemother.* **36**, 153–157.

Furman, P. A., Fyfe, J. A., St Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S., and Mitsuya, H. (1986). Phosphorylation of 3′-azido-3″-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**, 8333–8337.

Gao, W. Y., Shirasaka, T., Johns, D. G., Broder, S., and Mitsuya, H. (1993). Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. *J. Clin. Invest.* **91**, 2326–2333.

Gao, G., Orlova, M., Georgiadis, M. M., Hendrickson, W. A., and Goff, S. P. (1997). Conferring RNA polymerase activity to a DNA polymerase: A single residue in reverse transcriptase controls substrate selection. *Proc. Natl. Acad. Sci. USA* **94**, 407–411.

Giaquinto, C., Rampon, O., and De Rossi, A. (2006). Antiretroviral therapy for prevention of mother-to-child HIV transmisión: Focus on single-dose nevirapine. *Clin. Drug Invest.* **26**, 611–627.

Gonzales, M. J., Wu, T. D., Taylor, J., Belitskaya, I., Kantor, R., Israelski, D., Chou, S., Zolopa, A. R., Fessel, W. J., and Shafer, R. W. (2003). Extended spectrum of HIV-1 reverse transcriptase mutations in patients receiving multiple nucleoside analog inhibitors. *AIDS* **17**, 791–799.

Gotte, M., Li, X., and Wainberg, M. A. (1999). HIV-1 reverse transcription: A brief overview focused on structure-function relationships among molecules involved in the initiation of the reaction. *Arch. Biochem. Biophys.* **365**, 199–210.

Gotte, M., Arion, D., Parniak, M. A., and Wainberg, M. A. (2000). The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. *J. Virol.* **74**, 3579–3585.

Gu, Z., Gao, Q., Fang, H. S., Salomon, H., Parniak, M. A., Goldberg, E., Cameron, J., and Wainberg, M. A. (1994a). Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2′,3′-dideoxycytidine and 2′,3′- deoxythiacytidine. *Antimicrob. Agents Chemother.* **38**, 275–281.

Gu, Z., Fletcher, R. S., Wainberg, M. A., and Parniak, M. A. (1994b). The K65R mutant reverse transcriptase associated with HIV-1 resistance to 2′,3′-dideoxycytidine, 2′,3′-dideoxy-3′-thiacytidine and 2′,3′-dideoxyinosine shows reduced sensitivity to the specific dideoxynucleoside triphosphate inhibitors *in vitro*. *J. Biol. Chem.* **169**, 28118–28122.

Gu, Z., Arts, E. J., Parniak, M. A., and Wainberg, M. A. (1995). Mutated K65R recombinant reverse transcriptase of human immunodeficiency virus type 1 shows diminished chain termination in the presence of 2′,3′-dideoxycytidine 5,-triphosphate and other drugs. *Proc. Natl. Acad. Sci. USA* **92**, 2760–2764.

Gu, Z., Wainberg, M. A., Nguyen-Ba, N., L'Heureux, L., De Muys, J.-M., Bowlin, T. L., and Rando, R. F. (1999). Mechanism of action and in vitro activity of 1′,3′-dioxolanylpurine nucleoside analogues against sensitive and drug-resistant human immunodeficiency virus type 1 variants. *Antimicrob. Agents Chemother.* **43**, 2376–2382.

Hang, J. Q., Rajendran, S., Yang, Y., Li, Y., Wong Kai In, P., Overton, H., Parkes, K. B., Cammack, N., Martin, J. A., and Klumpp, K. (2004). Activity of the isolated HIV RNase H domain and specific inhibition by N-hydroxyimides. *Biochem. Biophys. Res. Commun.* **317**, 321–329.

Harrigan, P. R., Stone, C., Griffin, P., Najera, I., Bloor, S., Kemp, S., Tisdale, M., and Larder, B. (2000). Resistance profile of the human immunodeficiency virus type 1 reverse transcriptase inhibitor abacavir (1592U89) after monotherapy and combination therapy. CNA2001 Investigative Group. *J. Infect. Dis.* **181**, 912–920.

Harrigan, P. R., Salim, M., Stammers, D. K., Wynhoven, B., Brumme, Z. L., McKenna, P., Larder, B., and Kemp, S. D. (2002). A mutation in the 3 region of the human immunodeficiency virus type 1 reverse transcriptase (Y318F) associated with nonnucleoside reverse transcriptase inhibitor resistance. *J. Virol.* **76**, 6836–6840.

Harrigan, P. R., Wynhoven, B., Montaner, J., McKenna, P., and Bacheler, L. (2003). Mutations at reverse transcriptase codon 103: Phenotypic resistance to non-nucleoside reverse transcriptase inhibitor and clinical correlates. *Antivir. Ther.* **8**, S120.

Harris, D., Lee, R., Misra, H. S., Pandey, P. K., and Pandey, V. N. (1998). The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential in loading the p66 subunit on the template primer. *Biochemistry* **37**, 5903–5908.

Harris, K. S., Brabant, W., Styrchak, S., Gall, A., and Daifuku, R. (2005). KP-1212/1461, a nucleoside designed for the treatment of HIV by viral mutagenesis. *Antiviral Res.* **67**, 1–9.

Himmel, D. M., Sarafianos, S. G., Dharmasena, S., Hossain, M. M., McCoy-Simandle, K., Ilina, T., Clark, A. D., Jr., Knight, J. L., Julias, J. G., Clark, P. K., Krogh-Jespersen, K., Levy, R. M., *et al.* (2006). HIV-1 reverse transcriptase structure with RNase H inhibitor dihydroxy benzoyl naphthyl hydrazone bound at a novel site. *ACS Chem. Biol.* **1**, 702–712.

Hopkins, A. L., Ren, J., Esnouf, R. M., Willcox, B. E., Jones, E. Y., Ross, C., Miyasaka, T., Walker, R. T., Tanaka, H., Stammers, D. K., and Stuart, D. I. (1996). Complexes of HIV-1 reverse transcriptase with inhibitors of the HEPT series reveal conformational changes relevant to the design of potent non-nucleoside inhibitors. *J. Med. Chem.* **39**, 1589–1600.

Hossain, M. M., and Parniak, M. A. (2006). *In vitro* microbicide activity of the nonnucleoside reverse transcriptase inhibitor (NNRTI) UC781 against NNRTI-resistant human immunodeficiency virus type 1. *J. Virol.* **80**, 4440–4446.

Hsieh, J., Zinnen, S., and Modrich, P. (1993). Kinetic mechanism of the DNA-dependent DNA polymerase activity of HIV reverse transcriptase. *J. Biol. Chem.* **268**, 24607–24613.

Hsiou, Y., Ding, J., Das, K., Clark, A. D., Jr., Hughes, S. H., and Arnold, E. (1996). Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: Implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4**, 853–860.

Hsiou, Y., Das, K., Ding, J., Clark, A. D., Jr., Kleim, J. P., Rosner, M., Winkler, I., Riess, G., Hughes, S. H., and Arnold, E. (1998). Structures of Tyr188Leu mutant and wild-type HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor HBY 097: Inhibitor flexibility is a useful design feature for reducing drug resistance. *J. Mol. Biol.* **284**, 313–323.

Hsiou, Y., Ding, J., Das, K., Clark, A. D., Jr., Boyer, P. L., Lewi, P., Janssen, P. A., Kleim, J. P., Rosner, M., Hughes, S. H., and Arnold, E. (2001). The Lys103Asn mutation of HIV-1 RT: A novel mechanism of drug resistance. *J. Mol. Biol.* **309**, 437–445.

Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug resistance. *Science* **282**, 1669–1675.

Huang, W., Gamarnik, A., Limoli, K., Petropoulos, C. J., and Whitcomb, J. M. (2003). Amino acid substitutions at position 190 of human immunodeficiency virus type 1 reverse transcriptase increase susceptibility to delavirdine and impair virus replication. *J. Virol.* **77**, 1512–1523.

Isel, C., Ehresmann, C., Walter, P., Ehresmann, B., and Marquet, R. (2001). The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase. *J. Biol. Chem.* **276**, 48725–48732.

Iversen, A. K., Shafer, R. W., Wehrly, K., Winters, M. A., Mullins, J. I., Chesebro, B., and Merigan, T. C. (1996). Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. *J. Virol.* **70**, 1086–1090.

Jablonowski, H. (1995). Studies of zidovudine in combination with didanosine and zalcitabine. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **10**, S52–S56.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., *et al.* (1993). Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci USA* **90**, 6320–6324.

Jeffrey, J. L., Feng, J. Y., Qi, C. C., Anderson, K. S., and Furman, P. A. (2003). Dioxolane guanosine 5′-triphosphate, an alternative substrate inhibitor of wild-type and mutant HIV-1 reverse transcriptase: Steady state and pre-steady state kinetic analyses. *J. Biol. Chem.* **278**, 18971–18979.

Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992). Mechanism and fidelity of HIV reverse transcriptase. *J. Biol. Chem.* **267**, 25988–25997.

Kaushik, N., Harris, D., Rege, N., Modak, M. J., Yadav, P. S., and Pandey, V. N. (1997). Role of glutamine 151 of human immunodeficiency virus type 1 reverse transcriptase in RNA-directed DNA synthesis. *Biochemistry* **36**, 14430–14438.

Kerr, S. G., and Anderson, K. S. (1997). Pre-steady-state kinetic characterization of wild type and 3″-azido-3″-deoxythymidine (AZT) resistant human immunodeficiency virus type 1 reverse transcriptase: Implication of RNA directed DNA polymerization in the mechanism of AZT resistance. *Biochemistry* **36**, 14064–14070.

Klumpp, K., and Mirzadegan, T. (2006). Recent progress in the design of small molecule inhibitors of HIV RNase H. *Curr. Pharm. Des.* **12**, 1909–1922.

Klumpp, K., Hang, J. Q., Rajendran, S., Yang, Y., Derosier, A., Wong Kai In, P., Overton, H., Parkes, K. B., Cammack, N., and Martin, J. A. (2003). Two-metal-ion mechanism of RNA cleavage by HIV RNase H and mechanism based design of selective HIV RNase H inhibitors. *Nucleic Acids Res.* **31**, 6852–6859.

Kim, H. O., Schinazi, R. F., Nampalli, S., Shanmuganathan, K., Cannon, D. L., and Alves, A. J. (1993). 1, 3-Dioxolanylpurine nucleosides (2R, 4R) and (2R, 4S) with selective anti-HIV-1 activity in human lymphocytes. *J. Med. Chem.* **36**, 30–37.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.

Kroeger-Smith, M. B., Rouzer, C. A., Taneyhill, L. A., Smith, N. A., Hughes, S. H., Boyer, P. L., Janssen, P. A., Moereels, H., Koymans, L., and Arnold, E. (1995). Molecular modeling studies of HIV-1 reverse transcriptase nonnucleoside inhibitors: Total energy of complexation as a predictor of drug placement and activity. *Protein Sci.* **4**, 2203–2222.

Kuritzkes, D. R., Shugarts, D., Bakhtiari, M., Poticha, D., Johnson, J., Rubin, M., Gingeras, T. R., Kennedy, M., and Eron, J. J. (2000). Emergence of dual resistance to zidovudine and lamivudine in HIV-1- infected patients treated with zidovudine plus lamivudine as initial therapy. *J. Acquir. Immune Defic. Syndr.* **23**, 26–34.

Lacey, S. F., and Larder, B. A. (1994). Novel mutation (V75T) in human immunodeficiency virus type 1 reverse transcriptase confers resistance to 2′,3′-didehydro-2′,3′-dideoxythymidine in cell culture. *Antimicrob. Agents Chemother.* **38**, 1428–1432.

Larder, B. A., and Kemp, S. D. (1989). Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155–1158.

Larder, B. A., and Stammers, D. K. (1999). Closing in on HIV drug resistance. *Nat. Struct. Biol.* **6**, 103–106.

Lennerstrand, J., Stammers, D. K., and Larder, B. A. (2001). Biochemical mechanism of human immunodeficiency virus type 1 reverse transcriptase resistance to stavudine. *Antimicrob. Agents Chemother.* **45**, 2144–2146.

Lin, T. S., Schinazi, R. F., and Prusoff, W. H. (1987). Potent and selective in vitro activity of 3″-deoxythymidin-2′-ene (3′-deoxy-2′,3′- didehydrothymidine) against human immunodeficiency virus. *Biochem. Pharmacol.* **36**, 2713–2718.

Lin, P. F., Samanta, H., Rose, R. E., Patick, A. K., Trimble, J., Bechtold, C. M., Revie, D. R., Khan, N. C., Federici, M. E., and Li, H. (1994). Genotypic and phenotypic analysis of human immunodeficiency virus type 1 isolates from patients on prolonged stavudine therapy. *J. Infect. Dis.* **170**, 1157–1164.

Loeb, L., Essigmann, J., Kazazi, F., Zhang, J., Rose, K., and Mullins, J. (1999). Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc. Natl. Acad. Sci. USA* **96**, 1492–1497.

Majumdar, C., Abbotts, J., Broder, S., and Wilson, S. H. (1988). Studies on the mechanism of human immunodeficiency virus reverse transcriptase: Steady-state kinetics, processivity, and polynucleotide inhibition. *J. Biol. Chem.* **263**, 15657–15665.

Marcelin, A. G., Delaugerre, C., Wirden, M., Viegas, P., Simon, A., Katlama, C., and Calvez, V. (2004). Thymidine analogue reverse transcriptase inhibitors resistance mutations profiles and association to other nucleoside reverse transcriptase inhibitors resistance mutations observed in the context of virological failure. *J. Med. Virol.* **72**, 162–165.

Marcelin, A. G., Flandre, P., Pavie, J., Schmidely, N., Wirden, M., Lada, O., Chiche, D., Molina, J. M., and Calvez, V. (2005). Clinically relevant genotype interpretation of resistance to didanosine. *Antimicrob. Agents Chemother.* **49**, 1739–1744.

Margot, N. A., Isaacson, E., McGowan, I., Cheng, A. K., Schooley, R. T., and Miller, M. D. (2002). Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF. *AIDS* **16**, 1227–1235.

Masquelier, B., Race, E., Tamalet, C., Descamps, D., Izopet, J., Buffet-Janvresse, C., Ruffault, A., Mohammed, A. S., Cottalorda, J., Schmuck, A., Calvez, V., Dam, E., *et al.* (2001). Genotypic and phenotypic resistance patterns of human immunodeficiency virus type 1 variants with insertions or deletions in the reverse transcriptase (RT): Multicenter study of patients treated with RT inhibitors. *Antimicrob. Agents Chemother.* **45**, 1836–1842.

Mellors, J. W., Dutschman, G. E., Im, G.-J., Tramontano, E., Winkler, S. R., and Cheng, Y.-C. (1992). *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. *Mol. Pharmacol.* **41**, 446–451.

Meyer, P. R., Matsuura, S. E., So, A. G., and Scott, W. A. (1998). Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc. Natl. Acad. Sci. USA* **95**, 13471–13476.

Meyer, P. R., Matsuura, S. E., Mian, A. M., So, A. G., and Scott, W. A. (1999). A mechanism of AZT resistance: An increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol. Cell* **4**, 35–43.

Meyer, P. R., Matsuura, S. E., Schinazi, R. F., So, A. G., and Scott, W. A. (2000). Differential removal of thymidine nucleotide analogues from blocked DNA chains by human immunodeficiency virus reverse transcriptase in the presence of physiological concentrations of 2′-deoxynucleoside triphosphates. *Antimicrob. Agents Chemother.* **44**, 3465–3472.

Meyer, P. R., Lennerstrand, J., Matsuura, S. E., Larder, B. A., and Scott, W. A. (2003). Effects of dipeptide insertions between codons 69 and 70 of human immunodeficiency virus type 1 reverse transcriptase on primer unblocking, deoxynucleoside triphosphate inhibition, and DNA chain elongation. *J. Virol.* **77**, 3871–3877.

Mewshaw, J. P., Myrick, F. T., Wakefield, D. A. C. S., Hooper, B. J., Harris, J. L., McCreedy, B., and Borroto-Esoda, K. (2002). Dioxolane guanosine, the active form of the prodrug diaminopurine dioxolane, is a potent inhibitor of drug-resistant HIV-1 isolates from

patients for whom standard nucleoside therapy fails. *J. Acquir. Immune Defic. Syndr.* **29**, 11–20.

Miller, V., and Larder, B. A. (2001). Mutational patterns in the HIV genome and cross-resistance following nucleoside and nucleotide analogue drug exposure. *Antivir. Ther.* **6**, 25–44.

Miller, M. D., Anton, K. E., Mulato, A. S., Lamy, P. D., and Cherrington, J. M. (1999). Human immunodeficiency virus type 1 expressing the lamivudine-associated M184V mutation in reverse transcriptase shows increased susceptibility to adefovir and decreased replication capability *in vitro*. *J. Infect. Dis.* **179**, 92–100.

Miller, V., Ait-Khaled, M., Stone, C., Griffin, P., Mesogiti, D., Cutrell, A., Harrigan, R., Staszewski, S., Katlama, C., Pearce, G., and Tisdale, M. (2000). HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. *AIDS* **14**, 163–171.

Mitsuya, H., and Broder, S. (1988). Inhibition of infectivity and replication of HIV-2 and SIV in helper T-cells by 2′,3′-dideoxynucleocides *in vitro*. *AIDS Res. Hum.* Retroviruses **4**, 107–113.

Mitsuya, H., Weinhold, K. J., Furman, P. A., St Clair, M. H., Lehrman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985). 3″-Azido-3″-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**, 7096–7100.

Motakis, D., and Parniak, M. A. (2002). A tight-binding mode of inhibition is essential for the anti-HIV-1 microbicidal activity of nonnucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **46**, 1851–1856.

Murakami, E., Basavapathruni, A., Bradley, W., and Anderson, K. (2005). Mechanism of action of a novel viral mutagenic covert nucleotide: Molecular interactions with HIV-1 reverse transcriptase and host cell DNA polymerases. *Antiviral Res.* **67**, 10–17.

Naeger, L. K., Margot, N. A., and Miller, M. D. (2001). Increased drug susceptibility of HIV-1 reverse transcriptase mutants containing M184V and zidovudine-associated mutations: Analysis of enzyme processivity, chain-terminator removal and virus replication. *Antivir. Res.* **6**, 115–126.

Parkin, N., Chappey, C., Petropoulos, C., and Hellmann, N. (2003). HIV-1 reverse transcriptase mutations that suppress zidovudine resistance also increase *in vitro* susceptibility to tenofovir, but not stavudine. *Antivir. Ther.* **8**, S34.

Parniak, M. A., Min, K. L., Budihas, S. R., Le Grice, S. F. J., and Beutler, J. A. (2003). A fluorescence-based high throughput screening assay for inhibitors of HIV reverse transcriptase associated ribonuclease H activity. *Anal. Biochem.* **322**, 33–39.

Pata, J. D., Stirtan, W. G., Goldstein, S. W., and Steitz, T. A. (2004). Structure of HIV-1 reverse transcriptase bound to an inhibitor active against mutant reverse transcriptases resistant to other nonnucleoside inhibitors. *Proc. Natl. Acad. Sci. USA* **101**, 10548–10553.

Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D., Jr., Raag, R., Nanni, R. G., Hughes, S. H., and Arnold, E. (1995). Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase. *Biochemistry* **34**, 5351–5363.

Patton, D. L., Cosgrove-Sweeney, Y. T., Balkus, J. E., Rohan, L. C., Moncla, B. J., Parniak, M. A., and Hillier, S. H. (2007). Preclinical safety assessments of UC781 anti-HIV topical microbicide formulations. *Antimicrob. Agents Chemother.* **51**, 1608–1615.

Pelemans, H., Esnouf, R. M., Parniak, M. A., Vandamme, A. M., De Clercq, E., and Balzarini, J. (1998). A proline-to-histidine substitution at position 225 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) sensitizes HIV-1 RT to BHAP U-90152. *J. Gen. Virol.* **79**, 1347–1352.

Peliska, J. A., and Benkovic, S. J. (1992). Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* **258**, 1112–1118.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004). UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612.

Quan, Y., Motakis, D., Buckheit, R., Jr., Xu, Z. Q., Flavin, M. T., Parniak, M. A., and Wainberg, M. A. (1999). Sensitivity and resistance to (+)-calanolide A of wild-type and mutated forms of HIV-1 reverse transcriptase. *Antivir. Ther.* **4**, 203–209.

Reardon, J. E. (1992). Human immunodeficiency virus reverse transcriptase: Steady-state and pre-steady-state kinetics of nucleotide incorporation. *Biochemistry* **31**, 4473–4479.

Reardon, J. E. (1993). Human immunodeficiency virus reverse transcriptase: A kinetic analysis of RNA-dependent and DNA-dependent DNA polymerization. *J. Biol. Chem.* **268**, 8743–8751.

Reliquet, V., Ferre, V., Hascoet, C., Besnier, J. M., Bellein, V., Arvieux, C., Molina, J. M., Breux, J. P., Zucman, D., Rozenbaum, W., Allavena, C., and Raffi, F. (1999). Stavudine, didanosine and nevirapine in antiretroviral-naive HIV-1-infected patients. *Antivir. Ther.* **3**, 83–84.

Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D., and Stammers, D. (1995). High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Struct. Biol.* **2**, 293–302.

Ren, J., Milton, J., Weaver, K. L., Short, S. A., Stuart, D. I., and Stammers, D. K. (2000). Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. *Structure* **8**, 1089–1094.

Riddler, S. A., Anderson, R. E., and Mellors, J. W. (1995). Antiretroviral activity of stavudine (2″,3″-didehydro-3″-deoxythymidine, D4T). *Antiviral Res.* **27**, 189–203.

Richard, N., Salomon, H., Oliveira, M., Rando, R., Mansour, T., Gu, Z., and Wainberg, M. A. (2000). Selection of resistance-conferring mutations in HIV-1 by the nucleoside reverse transcriptase inhibitors (+/–) dOTC and (+/–) dOTFC. *Antivir. Chem. Chemother.* **11**, 359–365.

Richman, D. D. (2001). Antiretroviral activity of emtricitabine, a potent nucleoside reverse transcriptase inhibitor. *Antivir. Ther.* **6**, 83–88.

Richman, D., Shih, C.-K., Lowy, I., Rose, J., Prodanovich, P., Goff, S., and Griffin, J. (1991). Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88**, 11241–11245.

Richman, D. D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S. A., Sullivan, J., Cheeseman, S., Barringer, K., and Pauletti, D. (1994). Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *J. Virol.* **68**, 1660–1666.

Rigourd, M., Ehresmann, C., Parniak, M. A., Ehresmann, B., and Marquet, R. (2002). Primer unblocking and rescue of DNA synthesis by azidothymidine (AZT)-resistant HIV-1 reverse transcriptase. *J. Biol. Chem.* **277**, 18611–18618.

Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C., and Harrison, S. C. (1995). The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **92**, 1222–1226.

Sarafianos, S. G., Pandey, V. N., Kaushik, N., and Modak, M. J. (1995). Site-directed mutagenesis of arginine 72 of HIV-1 reverse transcriptase. *J. Biol. Chem.* **270**, 19729–19735.

Sarafianos, S. G., Das, K., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999a). Touching the heart of HIV-1 drug resistance: The fingers close down on the dNTP at the polymerase active site. *Chem. Biol.* **6**, 137–146.

Sarafianos, S. G., Das, K., Clark, A. D., Jr., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999b). Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc. Natl. Acad. Sci. USA* **96**, 10027–10032.

Sarafianos, S. G., Clark, A. D., Jr., Das, K., Tuske, S., Birktoft, J. J., Ilankumaran, P., Ramesha, A. R., Sayer, J. M., Jerina, D. M., Boyer, P. L., Hughes, S. H., and Arnold, E. (2002). Structures of HIV-1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. *EMBO J.* **21**, 6614–6624.

Sarafianos, S. G., Hughes, S. H., and Arnold, E. (2004). Designing anti-AIDS drugs targeting the major mechanism of HIV-1 RT resistance to nucleoside analog drugs. *Int. J. Biochem. Cell Biol.* **36**, 1706–1715.

Schinazi, R. F., Lloyd, R. M., Jr., Nguyen, M. H., Cannon, D. L., McMillan, A., Ilksoy, N., Chu, C. K., Liotta, D. C., Bazmi, H. Z., and Mellors, J. W. (1993). Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob. Agents Chemother.* **37**, 875–881.

Schmit, J. C., Van, L. K., Ruiz, L., Hermans, P., Sprecher, S., Sonnerborg, A., Leal, M., Harrer, T., Clotet, B., Arendt, V., Lissen, E., Witvrouw, M., *et al.* (1998). Multiple dideoxynucleoside analogue-resistant (MddNR) HIV-1 strains isolated from patients from different European countries. *AIDS* **12**, 2007–2015.

Schuurman, R., Nijhuis, M., van Leeuwen, R., Schipper, P., de Jong, D., Collis, P., Danner, S. A., Mulder, J., Loveday, C., and Christopherson, C. (1995). Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). *J. Infect. Dis.* **171**, 1411–1419.

Scott, L. J., and Perry, C. M. (2000). Delavirdine: A review of its use in HIV infection. *Drugs* **60**, 1411–1444.

Selmi, B., Boretto, J., Navarro, J. M., Sire, J., Longhi, S., Guerreiro, C., Mulard, L., Sarfati, S., and Canard, B. (2001). The valine-to-threonine 75 substitution in human immunodeficiency virus type 1 reverse transcriptase and its relation with stavudine resistance. *J. Biol. Chem.* **276**, 13965–13974.

Selmi, B., Deval, J., Alvarez, K., Boretto, J., Sarfati, S., Guerreiro, C., and Canard, B. (2003). The Y181C substitution in AZT-resistant human immunodeficiency virus type 1 reverse transcriptase suppresses the ATP-mediated repair of the AZTMP-terminated primer. *J. Biol. Chem.* **278**, 40464–40472.

Shafer, R. W., Kozal, M. J., Winters, M. A., Iversen, A. K., Katzenstein, D. A., Ragni, M. V., Meyer, W. A., Gupta, P., Rasheed, S., Coombs, R., and Merigan, T. C. (1994). Combination therapy with zidovudine and didanosine selects for drug-resistant human immunodeficiency virus type 1 strains with unique patterns of pol gene mutations. *J. Infect. Dis.* **169**, 722–729.

Shafer, R. W. (2002). Genotypic testing for human immunodeficiency virus type 1 drug resistance. *Clin. Microbiol. Rev.* **15**, 247–277.

Shaw-Reid, C. A., Munshi, V., Graham, P., Wolfe, A., Witmer, M., Danzeisen, R., Olsen, D. B., Carroll, S. S., Embrey, M., Wai, J. S., Miller, M. D., Cole, J. L., *et al.* (2003). Inhibition of HIV-1 ribonuclease H by a novel diketo acid, 4-[5-benzoylamino) thien-2-yl]-2,4-dioxobutanoic acid. *J. Biol. Chem.* **278**, 2777–2780.

Shirasaka, T., Yarchoan, R., O'Brien, M. C., Husson, R. N., Anderson, B. D., Kojima, E., Shimada, T., Broder, S., and Mitsuya, H. (1993). Changes in drug sensitivity of human immunodeficiency virus type 1 during therapy with azidothymidine, dideoxycytidine, and dideoxyinosine: An *in vitro* comparative study. *Proc. Natl. Acad. Sci. USA* **90**, 562–566.

Shulman, N., Zolopa, A. R., Passaro, D., Shafer, R. W., Huang, W., Katzenstein, D., Israelski, D. M., Hellmann, N., Petropoulos, C., and Whitcomb, J. (2001). Phenotypic hypersusceptibility to non-nucleoside reverse transcriptase inhibitors in treatment-experienced HIV-infected patients: Impact on virological response to efavirenz-based therapy. *AIDS* **15**, 1125–1132.

Simpson, D., and Tagliati, M. (1995). Nucleoside analogue-associated peripheral neuropathy in human immunodeficiency virus infection. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **9**, 153–161.

Sluis-Cremer, N., Dmitrienko, G. I., Balzarini, J., Camarasa, M. J., and Parniak, M. A. (2000a). Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-[Spiro [4″-amino-2″,2″-dioxo-1″,2″-oxathiole-5″,3″-[2″,5″-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]]]-3-ethylthymine. *Biochemistry* **39**, 1427–1433.

Sluis-Cremer, N., Arion, D., and Parniak, M. A. (2000b). Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). *Cell. Mol. Life Sci.* **57**, 1408–1422.

Sluis-Cremer, N., Arion, D., Kaushik, N., Lim, H., and Parniak, M. A. (2000c). Mutational analysis of Lys65 of HIV-1 reverse transcriptase. *Biochem. J.* **348**, 77–82.

Sluis-Cremer, N., Arion, D., and Parniak, M. A. (2002). Destabilization of the HIV-1 reverse transcriptase dimer upon interaction with N-acyl hydrazone inhibitors. *Mol. Pharmacol.* **62**, 398–405.

Sluis-Cremer, N., Arion, D., Parikh, U., Koontz, D., Schinazi, R., Mellors, J. W., and Parniak, M. A. (2005). The 3′-azido group is not the primary determinant of AZT responsible for the excision phenotype of AZT-resistant HIV-1. *J. Biol. Chem.* **280**, 29047–29052.

Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Chirino, A. J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1994). Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **91**, 3911–3915.

Smith, R. A., Loeb, L. A., and Preston, B. D. (2005). Lethal mutagenesis of HIV. *Virus Res.* **107**, 215–228.

Soudeyns, H., Yao, X.-J., Gao, Q., Belleau, B., Kraus, J. L., Nguyen-Bu, N., Spira, B., and Wainberg, M. A. (1991). Anti-humanimmunodeficiency virus type 1 activity and *in vitro* toxicity of 2″-deoxy-3″-thiacytidine (BCH-189), novel heterocyclic nucleoside analog. *Antimicrob. Agents Chemother.* **35**, 1386–1390.

Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995). Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**, 988–993.

Spence, R. A., Anderson, K. S., and Johnson, K. A. (1996). HIV-1 reverse transcriptase resistance to nonnucleoside inhibitors. *Biochemistry* **35**, 1054–1063.

Srinivas, R., and Fridland, A. (1998). Antiviral activities of 9-R-2 phosphonomethoxypropyl adenine (PMPA) and bis (isopropyloxymethylcarbonyl) PMPA against various drug-resistant human immunodeficiency virus strains. *Antimicrob. Agents Chemother.* **42**, 1484–1487.

Struble, K. A., Pratt, R. D., and Gitterman, S. R. (1997). Toxicity of antiretroviral agents. *Am. J. Med.* **102**, 65–77.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A. J., and Arnold, E. (1994). Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. *J. Mol. Biol.* **243**, 369–387.

Tisdale, M., Schulze, T., Larder, B. A., and Moelling, K. (1991). Mutations within the RNH domain of human immunodeficiency virus type 1 reverse transcriptase abolish virus infectivity. *J. Gen. Virol.* **72**, 59–66.

Tisdale, M., Kemp, S. D., Parry, N. R., and Larder, B. A. (1993). Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **90**, 5653–5656.

Tramontano, E. (2006). HIV-1 RNase H: Recent progress in an exciting, yet little explored, drug target. *Mini Rev. Med. Chem.* **6**, 727–737.

Tucker, T. J., Lumma, W. C., and Culberson, J. C. (1996). Development of nonnucleoside HIV reverse transcriptase inhibitors. *Meth. Enzymol.* **275**, 440–472.

Tuske, S., Sarafianos, S. G., Clark, A. D., Jr., Ding, J., Naeger, L. K., White, K. L., Miller, M. D., Gibbs, C. S., Boyer, P. L., Clark, P., Wang, G., Gaffney, B. L., *et al.* (2004). Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struct. Mol. Biol.* **11**, 469–474.

Valer, L., Martin-Carbonero, L., de Mendoza, C., Corral, A., and Soriano, V. (2004). Predictors of selection of K65R: Tenofovir use and lack of thymidine analogue mutations. *AIDS* **18**, 2094–2096.

Van Vaerenbergh, K., Van Laethem, K., Albert, J., Boucher, C. A., Clotet, B., Floridia, M., Gerstoft, J., Hejdeman, B., Nielsen, C., Pannecouque, C., Perrin, L., Pirillo, M. F., *et al.* (2000). Prevalence and characteristics of multinucleoside-resistant human immunodeficiency virus type 1 among European patients receiving combinations of nucleoside analogues. *Antimicrob. Agents Chemother.* **44**, 2109–2117.

Vivet-Boudou, V., Didierjean, J., Isel, C., and Marquet, R. (2006). Nucleoside and nucleotide inhibitors of HIV-1 replication. *Cell. Mol. Life Sci.* **63**, 163–186.

Wainberg, M. A., Drosopoulos, W. C., Salomon, H., Hsu, M., Borkow, G., Parniak, M., Gu, Z., Song, Q., Manne, J., Islam, S., Castriota, G., and Prasad, V. R. (1996). Enhanced fidelity of 3TC-selected mutant HIV-1 reverse transcriptase. *Science* **271**, 1282–1285.

Whitcomb, J. M., Parkin, N. T., Chappey, C., Hellmann, N. S., and Petropoulos, C. J. (2003). Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. *J. Infect. Dis.* **188**, 992–1000.

Winters, M. A., and Merigan, T. C. (2001). Variants other than aspartic acid at codon 69 of the human immunodeficiency virus type 1 reverse transcriptase gene affect susceptibility to nuleoside analogs. *Antimicrob. Agents Chemother.* **45**, 2276–2279.

Winters, M. A., Shafer, R. W., Jellinger, R. A., Mamtora, G., Gingeras, T., and Merigan, T. C. (1997). Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1 to 2 years. *Antimicrob. Agents Chemother.* **41**, 757–762.

Whitcomb, J. M., Huang, W., Limoli, K., Paxinos, E., Wrin, T., Skowron, G., Deeks, S. G., Bates, M., Hellmann, N. S., and Petropoulos, C. J. (2002). Hypersusceptibility to nonnucleoside reverse transcriptase inhibitors in HIV-1: Clinical, phenotypic and genotypic correlates. *AIDS* **16**, F41–F47.

Wu, J., Amandoron, E., Li, X., Wainberg, M. A., and Parniak, M. A. (1993). Monoclonal antibody mediated inhibition of HIV-1 reverse transcriptase polymerase activity: Interaction with possible nucleoside triphosphate binding domain. *J. Biol. Chem.* **268**, 9980–9985.

Yahi, N., Tamalet, C., Tourres, C., Tivoli, N., and Fantini, J. (2000). Mutation L210W of HIV-1 reverse transcriptase in patients receiving combination therapy. Incidence, association with other mutations, and effects on the structure of mutated reverse transcriptase. *J. Biomed. Sci.* **7**, 507–513.

Yang, H., Qi, X., Sabogal, A., Miller, M., Xiong, S., and Delaney, W. E., IV (2005). Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV. *Antivir. Ther.* **10**, 625–633.

Young, S. D., Britcher, S. F., Tran, L. O., Payne, L. S., Lumma, W. C., Lyle, T. A., Huff, J. R., Anderson, P. S., Olsen, D. B., and Carroll, S. S. (1995). L-743, 726 (DMP-266): A novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **39**, 2602–2605.

This page intentionally left blank

Hiroaki Mitsuya*,†, Kenji Maeda*, Debananda Das*, and Arun K. Ghosh‡

*The Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

†Department of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860–8556

‡Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907

Development of Protease Inhibitors and the Fight with Drug-Resistant HIV-1 Variants

I. Chapter Overview

The development of antiretroviral therapy for acquired immunodeficiency syndrome (AIDS) has witnessed one of the most dramatic progressions in the history of medicine. By the late 1980s, it had become apparent that combination chemotherapy with two nucleoside reverse transcriptase inhibitors (NRTIs) was more effective than NRTI monotherapy. However, only with the advent of protease inhibitors (PIs) in early 1990s, providing highly active antiretroviral therapy (HAART), significant clinical benefits became to be seen.

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56006-0

In this chapter, we discuss the principle and utility of development of PIs and the present challenges in the fight with emergence of PI-resistant HIV-1 variants.

II. Introduction

One can say that the development of antiretroviral therapy for AIDS has traced one of the most dramatic progressions in the history of medicine, showing combinations of rapid drug development, short-lived trends, and continuous evolution. In the latter half of 1980s, in the United States, efforts had been made to bring synergism to the basic research programs of the US government, private sectors, and academics on *Human immunodeficiency virus 1* (HIV-1), and to translate the basic findings into rapid development of novel therapeutics for AIDS. A focus of research on HIV-1 protease, the virally encoded enzyme has been targeted following the therapeutic success achieved by targeting at HIV-1 reverse transcriptase (Mitsuya and Erickson, 1999). Initially such an entirely new area of research was not financially well supported by industries. Moreover, the clinical utility of PIs, which had been designed using the knowledge of the molecular structure of protease, was not known. However, it had become apparent that combination chemotherapy with two NRTIs, zidovudine (azidothymidine, AZT) (Mitsuya *et al.*, 1985) and didanosine (dideoxyinosine, ddI) (Mitsuya and Broder, 1986; Yarchoan *et al.*, 1989a,b) was more effective than monotherapy as opposed to using the drugs sequentially (Yarchoan *et al.*, 1994). Between December 1995 and March 1996, three PIs, saquinavir (SQV), followed by ritonavir (RTV), and indinavir (IDV), were approved as prescription drugs for therapy of AIDS through the fast track approval mechanism by the US Food and Drug Administration (FDA) (Mitsuya and Erickson, 1999). Combination chemotherapy, with one of the PIs added to the combined NRTIs, produced sensational results in comparison to the clinical data that had been previously reported.

III. Targeting Viral Protease

A. Mechanism of Action of PIs

HIV-1 encodes a protease, also known as a proteolytic enzyme, which is responsible for the posttranslational processing of the viral products and is required for viral infectivity. Indeed, a mutation of the protease active site aspartic acids or chemical inhibition of the enzyme leads to the production of immature, noninfectious viral particles (Ghosh *et al.*, 2006a,b; Mitsuya and Erickson, 1999; Turk, 2006). The HIV-1 protease is an aspartyl protease that cleaves the HIV Gag and Gag-Pol polyproteins to generate structural

proteins and enzymes of the virus. This processing occurs late in the HIV life cycle during assembly and release from the infected cell and is an essential step for the formation of mature virus particles.

The dimeric HIV-1 protease consists of two identical monomer subunits of 99 amino acids and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′) (Fig. 1). The active site of the enzyme is unusual in that it is formed at the dimer interface and contains two conserved catalytic aspartic acid residues, one from each monomer. The substrate-binding cleft that surrounds the active site contains both hydrophobic and hydrophilic elements. Each monomer of the protease has a β-hairpin region (residues 45–56; Fig. 1) that overlaps to form a "flap" that extends over the binding cleft for the substrate. The flap is flexible enough to allow entry and exit of the polypeptide substrates and undergoes large localized conformational changes during the binding and release of inhibitors and substrates. Indeed, Hornak and their colleagues have shown using molecular dynamics simulation techniques that the unliganded HIV-1 protease flaps spontaneously open and reclose and that the flaps of the unliganded protease open to a much greater degree than observed in crystal structures and subsequently

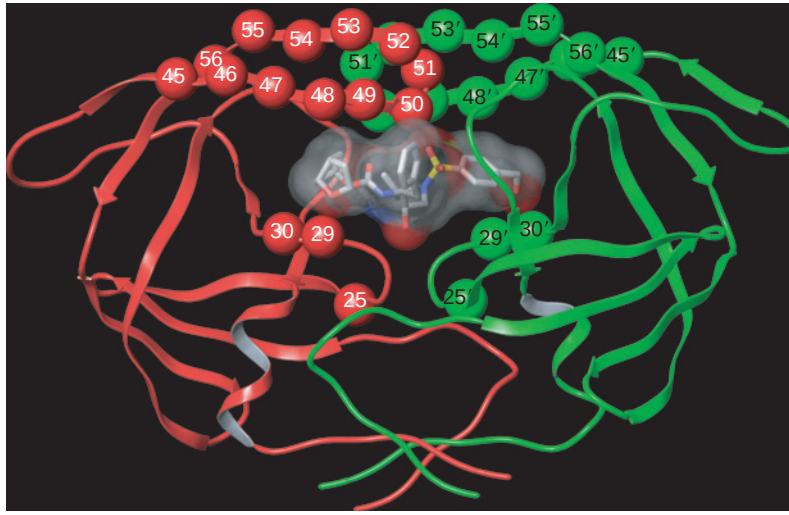


FIGURE 1 Structure of HIV-1 protease. The HIV protease consists of two identical 99 amino acid subunits and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′). Each monomer contributes amino acids (positions 45–56) to form a flap that extends over the substrate-binding cleft. The active site is covered by two β-hairpin structures or "flaps" that are highly flexible and undergo large localized conformational changes during the binding and release of inhibitors and substrates.

return to the semi-open state (Hornak *et al.*, 2006). For each substrate, three to four amino acids located on either side of the peptide bond cleavage site are utilized for binding to the substrate cavity of protease. Protease must cleave the immature HIV-1 polyprotein precursors, Gag and Gag-Pol, in at least nine different cleavage sites for maturation to occur (Jacobsen *et al.*, 1992). There is very little homology in the primary amino acid sequences of each of these cleavage sites. Instead, substrate specificity appears to be dictated by the secondary structure that remains conserved in each of the different cleavage sites.

Knowledge of the structure and functions of viral protease has led to the successful development of a wide variety of potent and chemically diverse inhibitors that have been designed using substrate- and structure-based approaches. The first PIs were designed in the early 1990s; those inhibitors were designed in such a way that the inhibitors fit exactly into the active site of the enzyme (Kempf *et al.*, 1990; Sommadossi, 1999). There are currently nine PIs approved for the treatment of HIV-1 infection (Fig. 2). All are competitive inhibitors that bind to the protease active site.

B. Protease Structures and Substrate-Based Inhibitors

In theory, antiviral drugs exert their effects by interacting with viral structural components, virally encoded enzymes, viral genomes, or specific host proteins such as cellular receptors, enzymes, or other factors required for viral replication (Mitsuya and Broder, 1987; Mitsuya and Erickson, 1999; Mitsuya *et al.*, 1990; Turk, 2006). In principle, any virus-specific steps in the replicative cycle of HIV-1, which differs from that in normal host cell function, can serve as a potential target for the development of antiretroviral therapy.

The close structural and functional relationships between retroviral and cellular aspartic proteases, together with knowledge of the HIV-1 protease cleavage site sequences on polyproteins, immediately opened an avenue of peptidomimetic substrate-based approaches that had been developed for designing inhibitors of human renin, an aspartic protease that has long been an important target for the design of antihypertensive agents. Substrate-based inhibitors are essentially peptide substrate analogues in which the scissile peptide bond has been replaced by a non-cleavable, transition-state analogue or isostere. Examples of this class of inhibitors include the first FDA-approved PI, SQV (Fig. 2), which essentially mimics the Phe-Pro cleavage site sequence (Roberts *et al.*, 1990).

C. Design of Symmetry-Based Inhibitors

With the understanding that HIV-1 protease is a twofold (C2) symmetric homodimer in which the active site is formed at the dimer interface and is

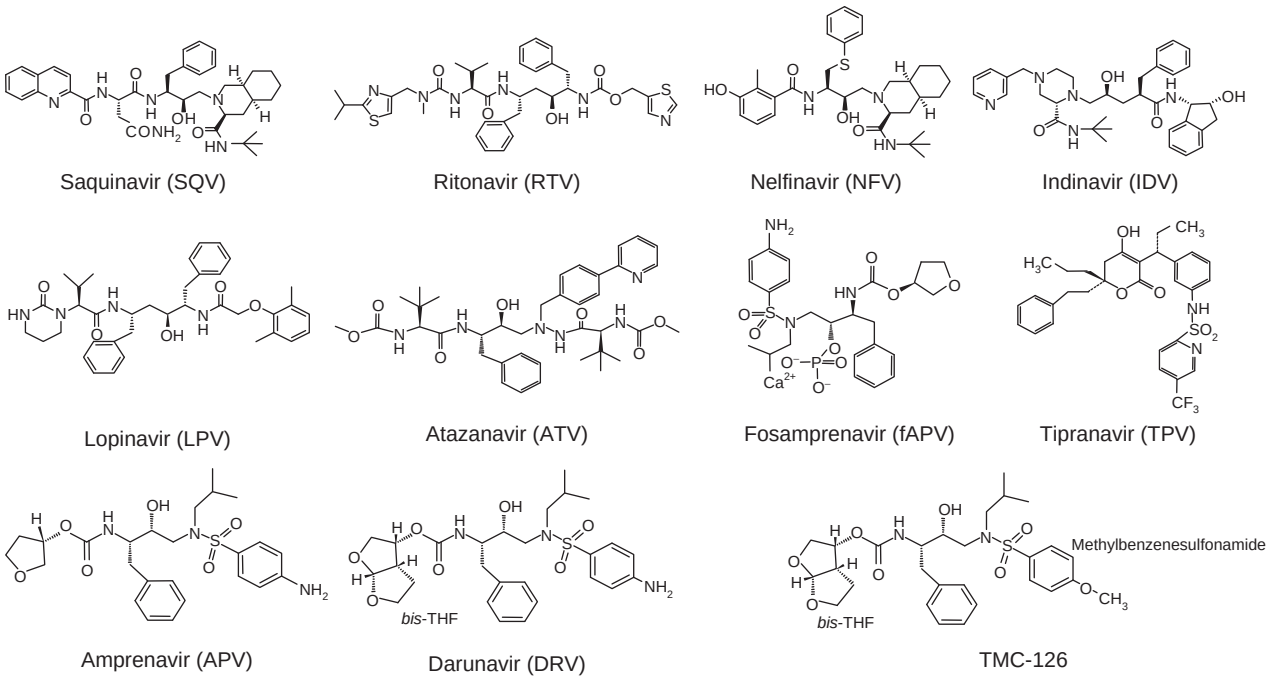


FIGURE 2 Clinically approved PIs. Structures of 10 PIs, thus far FDA-approved, are shown. Fosamprenavir is the prodrug for amprenavir. TMC-126 (not used in humans) is a prototype to darunavir.

composed of equivalent contributions of residues from each subunit came the realization that symmetry could be incorporated into the design of inhibitors for the HIV enzyme. Such designs represented a departure from traditional medicinal chemistry approaches to enzyme inhibitor designs (Erickson *et al.*, 1990; Kempf *et al.*, 1990). Examples for this type of PIs include RTV (Fig. 2).

D. Structure-Based PIs

As of today, well over 200 crystal structures have been solved and deposited in the Protein Data Bank (PDB) for various HIV-1 protease/inhibitor complexes—a testimony to the importance placed on structural information in the process of inhibitor design (Fitzgerald and Springer, 1991; Mitsuya and Erickson, 1999). Combined with medicinal chemistry and, in some cases, target-based screening efforts, these structural investigations have led to a structurally diverse compendium of inhibitors that include inhibitors like nelfinavir (NFV), that were derived solely using structure-based design methods, indinavir (IDV), and amprenavir (APV), the design of which was a blend of medicinal chemistry and structural insights (Fig. 2).

IV. The Role of PIs and Challenges in HAART

HAART, which typically exploits two reverse transcriptase inhibitors (RTIs) and one PI combined (or "boosted," *vide infra*) with RTV, has had a major impact on the AIDS epidemic in industrially advanced nations. However, no eradication of HIV-1 appears to be currently possible, in part, due to the viral reservoirs remaining in blood and infected tissues. Moreover, we have encountered a number of challenges in bringing about the optimal benefits of the currently available therapeutics of AIDS and HIV-1 infection to individuals receiving HAART (De Clercq, 2002; Siliciano *et al.*, 2004; Simon and Ho, 2003). They include (1) drug-related toxicities, (2) partial restoration of immunologic functions once individuals developed AIDS, (3) development of various cancers as a consequence of survival prolongation, (4) flame-up of inflammation in individuals receiving HAART or immune reconstruction syndrome (IRS), and (5) increased cost of antiviral therapy (Carr, 2003; Fumero and Podzamczer, 2003; Grabar *et al.*, 2006; Hirsch *et al.*, 2004; Little *et al.*, 2002).

Unlike the case for the majority of RTIs, most HIV-1 PIs had pharmacokinetic limitations. Poor oral absorption, serum–protein binding, and liver enzyme metabolism can eliminate the antiviral benefits of many otherwise highly potent PIs. PIs need to be ingested often and in large quantities to maintain effective antiviral concentrations in the blood. Furthermore, of the currently available antiviral drugs for HIV-1 infection, PIs are among the most effective, but they are costly and require complicated treatment regimens. Problematic

features of PIs are mostly inherent to their chemical natures: (1) high pill burden, (2) frequent dosing regimens, and (3) various side effects including lipodystrophy and dyslipidemia, although PIs remain an essential component of combination chemotherapy for both drug-naive and treatment-experienced patients with AIDS. It is worth noting that NRTIs are associated with critical adverse effects including mitochondrial toxicity. The role of PIs in HAART has been important and even PI-only regimens have been considered.

As soon as the first PIs were administered in humans, it was found that all PIs are inhibitors of the CYP3A4 system that is the major enzyme catabolizing most PIs and numerous other drugs. It catabolizes more than 50% of marketed drugs and is also frequently involved in drug–drug interactions (Overington *et al.*, 2006). RTV is by far the strongest inhibitor of CYP3A4, and SQV the weakest. Indeed, coadministration of low-dose RTV boosts the exposure of most PIs, which facilitates flexible dosing, including once daily dosing (*vide infra*).

V. "Boosting": A Critical Modification of Clinical Efficacy of PIs

Soon after the development of the first PIs, a problem inherent to this class of inhibitors was recognized, that is, poor pharmacokinetics, low maximum concentration (C_{max}), low plasma trough levels (C_{trough}), and short plasma half-life. The isoenzyme CYP3A4, a subunit of the cytochrome *P450* hepatic enzyme system, is mostly responsible for such poor pharmacokinetic parameters. RTV is by far the most potent inhibitor of the isoenzyme CYP3A4, and it was soon learned that concomitant administration of small doses of RTV with a PI allows "boosting" of the most important phamacokinetic parameters of almost all PIs (Kempf *et al.*, 1997). The unexpected but highly beneficial interactions between RTV and the other PIs have simplified otherwise complex regimens by reducing the frequency and number of pills to be administered, and in many cases by making dosing independent of food intake. Indeed, when boosted, PIs such as fosamprenavir and atazanavir can be taken only once a day. Moreover, boosting of certain PIs such as IDV or APV appears to make such PIs more effective against PI-resistant HIV-1 variants probably by elevating their plasma levels (Condra *et al.*, 2000). However, cautions should be used since plasma levels of PIs may decrease after long durations of treatment. For example, Gisolf *et al.* (2000) have reported that plasma levels of SQV even with RTV boosting dropped by 40% after a 10-month period of therapy. Thus, if there is any suspicion of reduced efficacy of boosted PI treatment after months of therapy, it is recommended that plasma levels of PI be examined and dose adjustments be made.

Until recently, only a limited set of data was available regarding the comparison of the clinical efficacy of each "boosted" PI-containing regimen. However,

there has recently been a wide range of settings where "boosted" PIs are being examined to compare clinical features of each member of PIs with or without other classes of antiretroviral agents. Indeed, the Department of Health and Human Services (DHHS) Guidelines (issued in October, 2006) (DHHS, 2006) recommends that first-line antiretroviral therapy be initiated with either an efavirenz-based regimen or the one containing twice-daily lopinavir/RTV, twice-daily fosamprenavir/RTV, or once-daily atazanavir/RTV. For example, the results of the KLEAN study involving antiretroviral-naive patients with HIV-1 infection have shown that lopinavir/RTV soft-gel capsules (SGC) *bid* and fosamprenavir/RTV *bid* with abacavir/lamivudine fixed dose combination (FDC) performed similarly with regard to virological and immunologic effects as well as adverse effects such as lipid elevations. Moreover, none of the patients with virological failure on either regimen had major PI resistance amino acid substitutions in their HIV-1 (Eron *et al.*, 2006). There are also new data showing that lopinavir/RTV SGC, when used as a once-a-day regimen combined with tenofovir and emtricitabine *qd*, maintains high levels of antiviral activity comparable to the twice-daily regimen in drug-naive patients (Johnson *et al.*, 2006). The results of the RESIST study have shown that tipranavir/RTV delivers better outcomes over comparator "boosted" PIs in highly treatment-experienced patients, although the role of the TPV/RTV combination in salvage therapy is likely to be modest due to its safety, pharmacokinetic issues (twice-daily regimen, etc.), and the near-future increasing availability of other more attractive antiretroviral drugs (Hicks *et al.*, 2006). Taken together, continuing evaluations of "boosted" PI-including regimens should certainly merit to improve the efficacy of HAART.

VI. Viral Resistance to PIs

As described above, HAART has had a major impact on the AIDS epidemic in industrially advanced nations; however, we have also encountered a number of challenges in bringing about the optimal benefits of the currently available therapeutics of AIDS to individuals receiving HAART (De Clercq, 2002; Siliciano *et al.*, 2004; Simon and Ho, 2003). Such limitations and flaws of HAART are worsened by the development of drug-resistant HIV-1 variants (Carr, 2003; Fumero and Podzamczer, 2003; Grabar *et al.*, 2006; Hirsch *et al.*, 2004; Little *et al.*, 2002). Table I illustrates mutations conferring high and intermediate resistance to currently approved PIs.

A. Emergence of Drug Resistance to PIs

A variety of drug resistance mechanisms are at play with PIs. The most important ones from a purely drug-binding standpoint are mutations in the

TABLE 1 Mutations Associated with Resistance to Currently Approved Protease Inhibitors

	Mutations conferring high/intermediate resistance								
Nelfinavir	D30N		M46I/L			V82A/T/F	I84V	N88S	L90M
Saquinavir				G48V		V82A	I84V		L90M
Indinavir			M46I/L			V82A/T/F/S	I84V		L90M
Ritonavir		L33F	M46I/L			V82A/T/F/S	I84V		L90M
Fosamprenavir[a]			M46I/L	I47V	I50V		I84V		L90M
Lopinavir		L33F	M46I/L	I47V	I50V	V82A/T/F/S	I84V		L90M
Atazanavir		L33F	M46I	G48V	I50V	V82A	I84V	N88S	L90M
Tipranavir		L33F	M46I			V82A/T/F/L	I84V		L90M
Darunavir[b]		L33F			I50V		I84V		

[a]Fosamprenavir is the prodrug of amprenavir; the latter is no longer manufactured.
[b]The mutations that are found in those with a diminished virological response to DRV/r in the POWER studies include V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V (De Meyer, 2006). However, it is not known yet as to which amino acid mutations are the major ones responsible for the apparent resistance to DRV/RTV.

active site of the protease enzyme that lead to loss of binding and hence diminished anti-HIV-1 activity of the inhibitor. Such mutations are necessary but not sufficient for the emergence of high-level resistance in the clinical setting. The reason for this appears to be that active site mutations alone result in only suboptimal resistance, which is consistent with biochemical studies on drug-resistant mutant proteases (Condra *et al*., 1995; Mitsuya and Erickson, 1999; Yin *et al*., 2006). The structures of protease, complexed with an inhibitor, allow us to attempt to rationalize the structural effects of drug resistance-conferring mutations on the interactions between the enzyme and inhibitor (Ghosh *et al*., 2006a,b; Mitsuya and Erickson, 1999). In some instances, these hydrogen bonds are mediated by bridging water molecules. The enzyme also contains a number of well-defined pockets (or subsites) in its active site region into which inhibitor's side chains protrude, resulting in tight binding interactions between the enzyme and the inhibitor. Since a similar pattern of hydrogen bonds are believed to be made for both substrates and peptidomimetic inhibitors, the specificity should reside in the pattern of largely nonpolar subsite interactions between the inhibitor and the enzyme side-chain atoms. Thus, mutations of specificity-determining residues that would directly interfere with inhibitor binding, but not with substrate processing, constitute an obvious mechanism

for resistance to PIs. Other resistance pathways might include nonactive site mutations that indirectly interfere with inhibitor binding through long-range structural perturbations of the active site, mutations that result in an enzyme with enhanced catalysis of stability, and cleavage site mutations that lead to enhanced processing by mutant enzymes (*vide infra*). Combinations of different mutations may also lead to additive, synergistic, and compensatory effects.

Mutations have been observed in nearly half of all possible positions in a monomer's 99 amino acids in response to drug-selection pressure (Ghosh *et al.*, 2006a,b; Mitsuya and Erickson, 1999; Molla *et al.*, 1996). Many of these mutations may presage the emergence of mutants in the clinical setting (Condra *et al.*, 1995). They can be classified as either active site or nonactive site mutations according to whether they occur inside or outside the inhibitor-binding subsites and directly contact the inhibitor (*vide infra*). Certain amino acid substitutions, common or not, exist in virus in a normal environment apparently having no substantial impact on fitness, are seen without regard to antiviral therapy in certain viral isolates, and termed polymorphism (e.g., lysine or glutamate at RT codon 122) (Kavlick and Mitsuya, 2001). Such polymorphisms might also coincidentally convey fitness on the virus in an altered environment, for example, improving virus fitness under selective drug pressure by conferring some degree of resistance. It can be stated that such a virus possesses *natural resistance* and can therefore negatively impact anti-HIV-1 therapy. When HIV-1 variants resistant to an NRTI, 2′-β-fluoro-2′,3′-dideoxyadenosine (FddA or lodenosine), *in vitro* (HIV-$1_{FddA}{}^{R}$), all clones derived from HIV-$1_{FddA}{}^{R}$ contained Pro119Ser and Leu214Phe substitutions, and an infectious clone containing Pro119Ser and Leu214Phe generated by site-directed mutagenesis confirmed phenotypic resistance to FddA (Tanaka *et al.*, 1997). However, clonal subpopulations of the wild-type HIV-1 (HIV-1_{LAI}) used in the selection also possessed the Pro119Ser (23%) and Leu214Phe (69%) substitutions (Tanaka *et al.*, 1997). These data suggest that certain natural viral polymorphisms may confer resistance and that such virus can expand under selective drug pressure.

For the latest information on genotypic and phenotypic HIV-1 drug resistance, visit http://hivdb.stanford.edu/pages/genotype-phenotype.html or http://www.hiv.lanl.gov/content/hiv-db/mainpage.html

B. Primary and Secondary Mutations

Drug resistance mutations that surround the active site usually interfere with the binding of a PI to the protease and are referred to primary mutations (Table I; Fig. 4). Because of their locations near the substrate-binding cleft, these mutations affect processing of the natural substrates as well and, therefore, often confer a fitness cost to HIV-1. Primary mutations that

interfere with PI binding, located distant to the active site, have also been documented (i.e., Leu90Met). However, most mutations that are not within the substrate-binding cavity do not affect inhibitor binding *per se* and do not confer resistance by itself (without other primary mutations), but compensate for the deleterious effects on enzymatic activity caused by primary mutations. These compensatory mutations are referred to as secondary mutations. Thus, high-level drug resistance to PIs requires the stepwise accumulation of multiple primary and secondary mutations to generate a protease capable of discriminating inhibitors from natural substrate and yet maintaining adequate catalytic efficiency needed for virus replication.

C. Active Site Mutants

The first described resistance mutation for HIV-1 protease was a Val82Ala mutation that was selected using a symmetric diol inhibitor (Otto *et al*., 1993). Since then, resistance mutations have been observed in each of the unique specificity pockets, S3, S2, S1 and, by symmetry, S1′, S2′, and S3′. However, only a subset of all residues that constitute a particular subsite mutates in response to a particular drug. The structural effects of mutations on drug binding have been modeled using the crystal structures of the appropriate wild-type enzyme–inhibitor complexes, and used to rationalize the effects of specific mutations on drug-binding affinities (Markowitz *et al*., 1995; Otto *et al*., 1993).

Most of the subsite mutations, such as Ile84Val and Val82Ala (or Ile or Phe), affect hydrophobic and van der Waals interactions and can be considered to be "packing" mutants somewhat analogous to hydrophobic mutations in a protein core (Markowitz *et al*., 1995). Crystallographic analyses of HIV-1 protease/inhibitor complexes show that most of the surface of an inhibitor and its immediate protein environment are solvent inaccessible (see more about Val82Ala substitution in Section VI). Some mutations, such as Val82Ile, are more effective when combined with second active site mutations such as Val32Ile (Kaplan *et al*., 1994). Other mutations can affect electrostatic interactions. While numerous crystal structures of wild-type HIV protease-inhibitor complexes have been published, crystal structures of mutant HIV protease-inhibitor complexes have also recently emerged in the literature.

D. Nonactive Site Mutants

While the precise structural mechanism of drug resistance can often be pinpointed for active site mutations that directly affect inhibitor binding, the evaluation of nonactive site mutants is more challenging and there may be several different mechanisms at work. Some mutations might act in concert with active site mutations by compensating for a functional deficit caused by the latter. For example, the defective Arg8Glu mutation is found

almost exclusively in combination with one or more mutations outside the active site region, such as Met46Ile (Ho *et al.*, 1994; see Fig. 4 for the site of Met-46). Mutations of Met-46 to Ile, Leu, or Phe are often found in the presence or absence of other active site mutations, such as Val82Ile, Ala or Phe, and Ile84Val. Met-46 is in the flap of HIV protease and recent molecular dynamic simulations on flap movement have shown that the Met46Ile mutant exhibits a markedly different dynamical behavior than the wild-type enzyme (Collins *et al.*, 1995), and presumably alters enzyme kinetics. However, a role for Met-46 in polyprotein–substrate recognition is also possible.

Other nonactive site mutants may indirectly alter the structure of the active site region. Many of these nonactive site mutations are found in multiple combinations with one or more active site mutations. In some cases, the introduction of nonactive site mutations alone does not lead to a marked or even measurable reduction in inhibitor binding, in contrast to the case for all known active site mutations. However, the fact that certain mutations are only observed in the presence of drug means that they must by definition provide the virus with some selective replication advantage. At least one engineered HIV-1 protease mutant, Gly48Tyr, exhibited greater catalytic efficiency than the wild-type enzyme toward artificial peptide substrates (Lin *et al.*, 1995).

E. Cleavage Site Mutants

Additional mutations in the HIV-1 genome have been found that do not lie within the protease enzyme, but are instead located near the cleavage sites of Gag-Pol and Gag substrates. These mutations are also secondary mutations that compensate for the reduced catalytic efficiency caused by primary protease mutations (Cote *et al.*, 2001; Doyon *et al.*, 1996; Tamiya *et al.*, 2004; Zhang *et al.*, 1997). Since active site mutations are thought to alter the rate of one or more cleavages that must occur during viral maturation, one can think that compensating mutations in the cleavage sites on the Gag or Gag-Pol polyproteins might render them better substrates for particular mutant enzymes. Studies by Doyon *et al.* (1996) have identified a mutation in the p1/p6 Gag polyprotein cleavage site (Leu449Phe) substitution that can synergize with the Ile84Val mutant to produce a virus with 350- and 500-fold decreased sensitivity to substrate-based PIs BILA 1906 BS and BILA 2185 BS, respectively. The mutation altered the p1/p6 cleavage site from Phe-Leu to Phe-Phe. Indeed, a synthetic peptide containing the Phe-Phe cleavage site was cleaved at higher catalytic efficiency by the Ile84Val HIV mutant protease than the corresponding peptide with the wild-type sequence. Cleavage site mutations at the p7/p1 cleavage site have also been

observed in breakthrough virus isolated from patients on IDV therapy (Zhang *et al*., 1997).

F. Noncleavage Site Mutants

In addition to the cleavage site mutations described above, mutations can also be seen in noncleavage sites in Gag-Pol polyproteins. For instance, multiple amino acid substitutions (e.g., Leu75Arg, His219Gln/Pro, and Val390Asp/Val390Ala) have been identified at noncleavage sites of Gag proteins, which emerge on long-term exposure to a PI(s) and are indispensable for HIV-1 replication in the presence of such a PI(s). These mutations affect Gag functions without affecting Gag's cleavage sensitivity to protease (Gatanaga *et al*., 2002). For instance, His219Gln and His219Pro represent polymorphic amino acid residues; however, these substitutions confer on HIV-1 replication advantage in a unique way (Gatanaga *et al*., 2006). Both human $CD4^{+}$MT-2 and H9 cell lines contain high levels of cyclophilin-A, and the cyclophilin-A content of virions generated in these cells is far greater than that in human peripheral blood mononuclear cells. Such high cyclophilin-A-containing virions limit the replication of HIV-1 containing wild-type His-219. The His219Gln and His219Pro substitutions reduce cyclophilin-A incorporation into virions and potentiate viral replication.

G. Insertions in Gag-Pol Polyproteins

The addition of certain amino acids can also contribute to the development of viral resistance. Winters *et al.* (1998) identified a 6-base pair insert between codons 69 and 70 of the RT gene in HIV-1 isolated from multiple NRTI-treated patients and conducted elegant site-directed mutagenesis studies, showing that the insert alone confers on HIV-1 reduced susceptibility to multiple NRTIs. Peters *et al.*(2001) have also identified duplication of a proline-rich motif, Ala-Pro-Pro (APP) in the PTAP motif of the Gag protein in HIV-1 variants isolated from patients with AIDS receiving NRTI(s) including ddI, d4T, AZT, 3TC, and have shown that this addition could improve the viral assembly and packaging at membrane locations, resulting in increased infectivity and viral resistance to NRTIs.

Tamiya *et al.* (2004) have also identified unique insertions (TGNS, SQVN, AQQA, SRPE, APP, and/or PTAPPA) near the p17/p24 and p1/p6 Gag cleavage sites in addition to the known multiple amino acid substitutions within the protease in full-length molecular infectious multidrug-resistant (MDR) HIV-1 clones generated from HIV-1 variants isolated from patients with AIDS who had received long-term antiviral therapy. Such inserts mostly compromise the enzymatic functions of the wild-type protease; however, they restore the Gag processing by the mutant protease and enable PI-resistant HIV-1 variants to remain replication competent.

VII. PIs with Activity Against Drug-Resistant HIV-1

A. Mutations That Allow Discrimination of PIs from Natural Substrates

Each PI tends to select for particular primary mutation(s) ("signature" mutations) and subsequent secondary mutations both *in vitro* and *in vivo* (Markowitz *et al.*, 1995; Otto *et al.*, 1993; Yusa and Harada, 2004). Nonetheless, many primary mutations are capable of causing cross-resistance to multiple PIs, even though they tend only to emerge during therapy with specific inhibitors (Hertogs *et al.*, 2000; Kemper *et al.*, 2001; Tisdale *et al.*, 1995; Watkins *et al.*, 2003). All PIs are shorter than the natural substrates and contain hydrophobic moieties that interact with S2-S2′ subsites (Fig. 3 for darunavir). Thus, although PIs may be chemically unique from each other, they occupy a similar space within the protease-binding cavity, which explains at least in part how individual mutations may cause PI cross-resistance.

Structural analysis of primary mutations has formed the basis for our current understanding of PI resistance at the molecular level. One such mutation is Val82Ala, originally described after selection with RTV or IDV (Condra *et al.*, 1995; Deeks *et al.*, 1998; Molla *et al.*, 1996). This mutation is capable of conferring HIV resistance to a number of PIs, particularly early generation compounds. Crystal structures of protease containing Val82Ala complexed with either natural substrates or a PI were compared

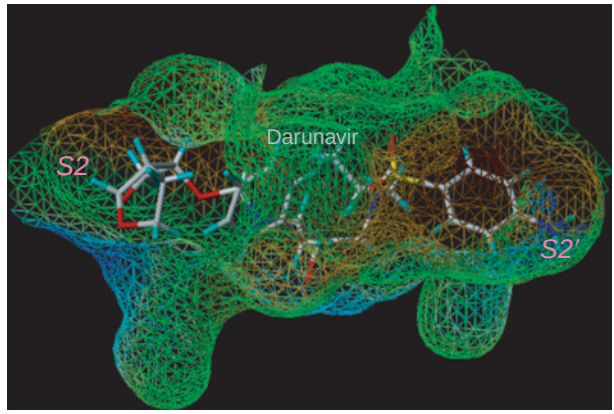


FIGURE 3 Darunavir bound in the hydrophobic cavity within protease. Hydrophobic cavity within protease with darunavir (DRV/TMC114; PDB ID 1S6G) is shown. Brown and green regions are lipophilic while the blue regions are hydrophilic (determined using MOLCAD). The S2 and S2′ subsites are indicated. The figure was generated using Sybyl 7.0 (Tripos, Inc.).

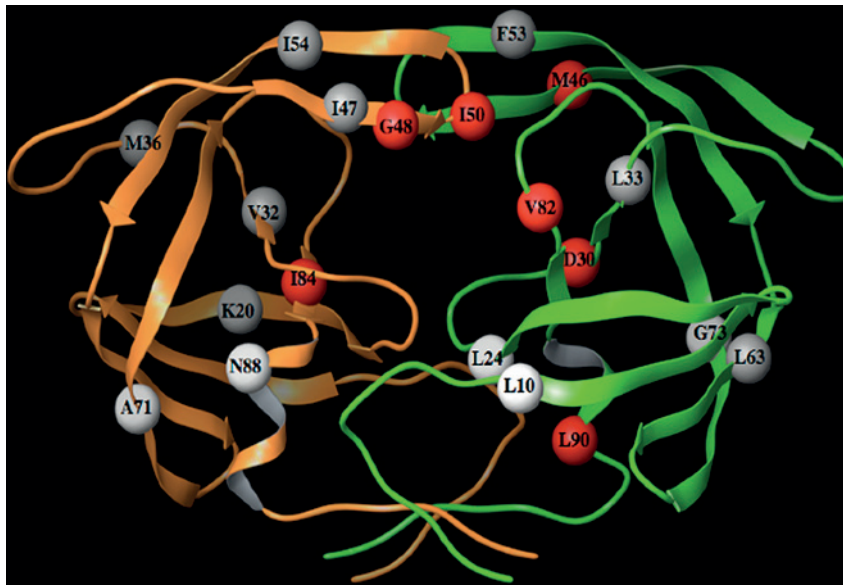


FIGURE 4 Locations of amino acid substitutions associated with drug resistance to PIs. Structure of protease homodimer with positions of amino acid residues associated with clinical resistance to current PIs is indicated. Primary and secondary mutations are indicated with red and white spheres, respectively. The protease monomers are shown in green and orange ribbons. Mutations are shown on only one monomer for clarity. The figure was generated using Maestro version 7.5.

(these enzymes also contained Asp25Asn, a mutation that inactivated the enzyme to prevent cleavage of the substrate but did not appear to affect hydrogen bonding between protease and ligand) (Prabu-Jeyabalan *et al.*, 2003). Val82Ala results in significant changes in the crystal structures of protease complexed to a PI, including changes in the flap position and subsequent disruption of hydrogen bonding, as well as the loss of van der Waals interactions between mutant protease and a PI. On the other hand, crystal structures between natural substrate peptides complexed to either wild-type protease or Val82Ala mutant protease have not demonstrated significant changes. Molecular interactions between protease and the longer natural substrates consist mainly of extensive backbone–backbone hydrogen bonds as well as more extensive van der Waals interactions (Prabu-Jeyabalan *et al.*, 2000, 2002, 2003). This suggests that the Val82Ala mutation, which decreases the size of the side chain, has little effect on substrate binding, but has a much greater detrimental effect on PI binding.

Others have shown that MDR protease with mutations at multiple positions (amino acids Leu-10, Met-36, Met-46, Ile-54, Leu-63, Ala-71, Val-82, Ile-84, and Leu-90) has an expanded active site cavity (Logsdon *et al.*, 2004).

Again, the binding of PIs to this MDR protease was noticeably different than binding to wild-type protease. Although the crystal structure of this MDR protease with natural substrates was not assessed, this work provides further insight into the structural effects of multiple protease resistance mutations.

To further understand the difference between substrate binding and PI binding to protease, an analysis of the structures of eight different inhibitors complexed to protease has been conducted. King *et al.* (2004a) demonstrated that despite the chemical differences of the PIs, all compounds occupied a similar volume within the active site cavity that is termed the "inhibitor envelope." If the inhibitor envelope was compared with the "substrate envelope," the space within the protease that is occupied by a natural substrate, the inhibitors protrude from the substrate envelope in very distinct locations. At these positions, PIs may have van der Waals interactions with amino acid positions such as Gly-48, Ile-50, Val-82, and Ile-84. It is known that mutations at these residues result in PI primary resistance (Table I, Fig. 4), and therefore, these mutations likely disrupt PI and protease molecular associations. In contrast, these same mutations have little effects on natural substrates that do not make molecular interactions at these amino acid positions (King *et al.*, 2004a).

B. Development of PIs with Activity Against Drug-Resistant HIV

Of the currently approved PIs, APV fits predominantly within the substrate envelope (King *et al.*, 2004b). The mutation profile for APV is also different compared with that for other PIs, providing more evidence that PIs that have greater resemblance to natural substrates will be less affected by primary mutations selected by first generation PIs. A conceptually intriguing structure-based design of PIs targeting active site protein backbone or "backbone binding" has resulted in inhibitors with impressive drug resistance profiles (Ghosh *et al.*, 2006c). Structural analysis revealed that while mutations occur throughout the protease, the backbone conformation is surprisingly conserved, especially in the active site (Ghosh *et al.*, 2006a). Because mutations of backbone atoms of the protease cannot occur, disruption of these bonds is more difficult compared with hydrogen bonds that many PIs form between amino acid sidechains, which can be affected by substitution mutations. Further development of inhibitors successfully exploiting these elements has resulted in PIs with significant activity against MDR HIV.

C. Design Rationale of Darunavir

APV contains an interesting tetrahydrofuranyl (3*S*-THF) urethane moiety as the P2 ligand (Fig. 2) (Kim *et al.*, 1995). The importance and potency enhancing effect of the 3(*S*)-THF ring was shown in inhibitors

containing both hydroxyethylene as well as hydroxyethylamine isostere (Ghosh *et al.*, 1993). The THF ring oxygen forms a weak hydrogen bond with the residue in the S2-region of the protease active site upon drug binding. Incorporation of a stereochemically defined 3(*R*), 3a(*S*), 6a(*R*)-*bis*-tetrahydrofuranyl (*bis*-THF) urethane in the hydroxyethylamine isostere led to two PIs, TMC-126 and darunavir (DRV, TMC-114) (Fig. 2) (Ghosh *et al.*, 1998, 2001, 2002). TMC-126 differs from darunavir due to a replacement of 4-aminobenzenesulfonamide with 4-methoxybenezenesulfonamide.

The critical *bis*-THF ligand was designed and developed to make extensive interactions with the residues at the S2-region and specifically target and maximize "backbone-binding" interactions (Ghosh *et al.*, 1994, 1996, 1998, 2006b). Structural analysis indeed revealed that the *bis*-THF derivative effectively hydrogen bonds to the NH of the Asp-29 and Asp-30 as shown (Fig. 5) (Koh *et al.*, 2003; Tie *et al.*, 2004). Both drugs have increased activity against both wild-type as well as clinical isolates containing multi-PI-resistant mutations (De Meyer *et al.*, 2005; Koh *et al.*, 2003; Yoshimura *et al.*, 2002). Furthermore, based on the analysis of DRV, the larger *bis*-THF rings of TMC-126 and DRV protrude slightly more from the substrate envelope

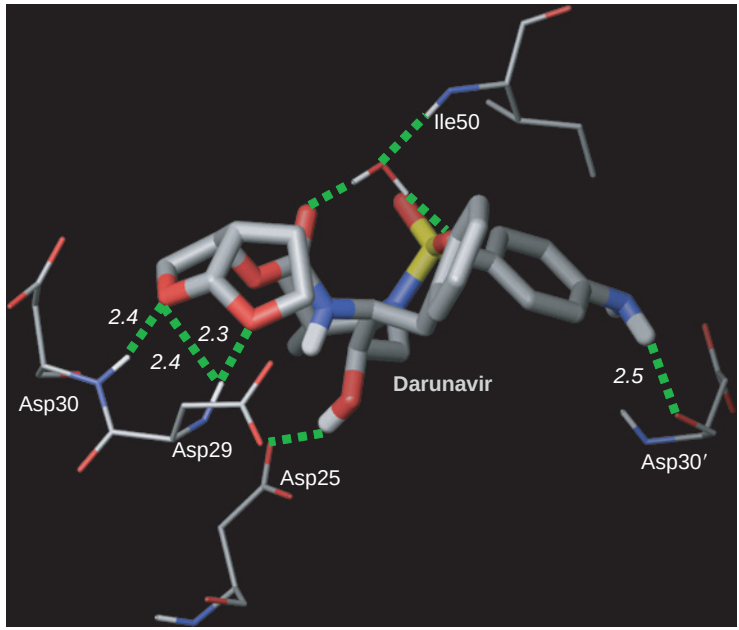


FIGURE 5 Hydrogen bond interactions of darunavir with protease catalytic sites. Hydrogen bond interactions of darunavir with Asp-29 and Asp-30 in the S2 subsite, and Asp-30′ in the S2′ subsite. The hydrogen bonds are shown in green broken lines. The figure was generated using Maestro version 7.5.

compared with the THF ring of APV (King *et al.*, 2004b). Although the THF ring of APV is able to form hydrogen bonds with the backbone carboxylate oxygen of Asp-30, the interaction is relatively weak. In contrast, *bis*-THF makes strong hydrogen bonds with the main chain atoms of Asp-29 and Asp-30 in the S2 subsite of the protease. Furthermore, the *bis*-THF moiety fills the hydrophobic pocket in the S2-region more effectively than the THF ring of APV. This may account for a tenfold increase in activity of TMC-126 and DRV against a panel of clinical isolates with various combinations of protease resistance mutations (Koh *et al.*, 2003; Surleraux *et al.*, 2005b).

Attempts to design compounds capable of further exploiting these critical interactions with the main chains of Asp-29 and Asp-30 in the S2 subsite are currently underway (Ghosh *et al.*, 2005). On the opposite side of these same inhibitor molecules, it has been reported that the P2′ substituents such as the 4-aminobenzenesulfonamide of APV and DRV, or the 4-methoxybenezenesulfonamide of TMC-126, also introduce hydrogen bonding with the backbone carboxyl of Asp-30′ in the S2′ subsite (Fig. 3) (Surleraux *et al.*, 2005b). Compounds designed to optimize this interaction have also demonstrated potent *in vitro* activity against PI-resistant HIV-1 variants (Surleraux *et al.*, 2005a). Maximization of hydrogen bond interactions between the protease backbone and TMC-126 or DRV results in highly favorable enthalpic contributions that drive inhibitor binding. This differs from first generation PIs (i.e., NFV, SQV, and IDV) that have unfavorable enthalpic interactions with protease. Binding of these PIs to protease was entropically driven as a result of the burial of hydrophobic residues of these compounds. Thus, mimicking the backbone hydrogen bonding of natural substrates in at least two separate subsites of protease has yielded more thermodynamically adaptable PIs capable of overcoming protease resistance conferred by amino acid substitutions (King *et al.*, 2004b; Ohtaka and Freire, 2005).

Katlama and her colleagues compared the efficacy and safety of multiple doses of DRV plus low-dose RTV (DRV/r) with investigator-selected control PI(s) (CPI[s]) in a phase IIb randomized POWER 1 clinical trial (Katlama *et al.*, 2007). This involved 318 patients with one or more primary PI mutation and HIV-1 RNA>1000 copies/ml, receiving optimized background therapy, plus DRV/r 400/100 mg *qd*, 800/100 mg *qd*, 400/100 mg *bid* or 600/100 mg *bid*, or CPI(s). DRV/r 600/100 mg *bid* demonstrated the highest virological and immunological responses (Katlama *et al.*, 2007). Adverse event incidence was similar between treatments: headache and diarrhea were more common with CPI(s). All patients receiving DRV/r were switched for the ongoing open-label phase of the trial.

D. HIV-1 Resistance to Darunavir

As mentioned above, APV selects for a unique pattern of protease resistance mutations compared with first generation PIs and this holds true for the structurally similar TMC-126 and DRV. TMC-126 resistance

appears to be mediated by a novel mutation, Asp28Ser, along with subsequent acquisition of Ile50Val (Yoshimura *et al*., 2002). Although Ile50Val has been demonstrated to confer primary resistance on APV in clinical isolates, Ala28Ser has not been described yet as a common protease resistance mutation, likely because of the effect this mutation has on the catalytic efficiency of protease (Hong *et al*., 1998; Yoshimura *et al*., 2002).

Computational modeling analysis does not indicate that TMC-126 has hydrogen bond interactions with either the backbone or side chain of Ala-28, which suggests that the reduction in potency of TMC-126 is due to either steric hindrance caused by the larger serine of A28S, or possibly due to unfavorable solvation energy effects during binding (Yoshimura *et al*., 2002). Although the pharmacokinetic properties of TMC-126 were not suitable for further clinical development, the related compound DRV exerts significant activity against multi-PI-resistant clinical HIV-1 isolates and has favorable pharmacokinetics (Arasteh *et al*., 2005; De Meyer *et al*., 2005; Koh *et al*., 2003); DRV has been approved as a prescription drug for treatment of those who do not respond to any other existing antiviral regimens in June 2006. Despite the chemical similarities of TMC-126 and DRV, Ala28Ser has not been described yet after DRV selection (Koh and Mitsuya, unpublished data). The reasons for this are unclear at this point. Instead, *in vivo* resistance with strains harboring Arg41Thr and Lys70Glu has been identified (De Meyer *et al*., 2005). Isolates harboring these two mutations were found to have 8-to 10-fold resistance to DRV, 20-fold resistance to SQV, and 6-fold resistance to lopinavir. Otherwise, resistance remained less than 4-fold for all other first generation PIs. The molecular mechanisms that allow Arg41Thr and Lys70Glu to confer DRV resistance is also currently unknown, as site-directed mutants carrying one or both of these mutations show no reduction in sensitivity to other PIs tested (De Meyer *et al*., 2005). Nonetheless, there appears to be a higher genetic barrier to the emergence of resistance to both TMC-126 and DRV, and both drugs have been shown to maintain potent activity against multi-PI-resistant strains, suggesting that their unique interactions with HIV protease can provide the framework for developing subsequent generations of PIs. It is of note that another *bis*-THF containing PI, brecanavir (GW640385), has shown activity against both wild-type and drug-resistant HIV (Ward *et al*., 2005). *In vitro* selection of HIV-1 with brecanavir resulted in the emergence of the novel A28S mutation seen initially with TMC-126 (Yates *et al*., 2004). Unfortunately, in late 2006 clinical trials of brecanavir were terminated due to the difficulty of formulation.

Profiles of HIV-1 resistance to DRV have now been gradually accumulated. De Meyer *et al*. (2005) selected the wild-type HIV-1_{LAI} with DRV in test tubes. Selection of resistant HIV-1 with other PIs was readily possible and resulted in the emergence of strains carrying known PI resistance-associated mutations. The concentrations of the current PIs could be readily increased to 1 μM, still allowing for virus replication. In contrast, DRV concentration could not be increased beyond 200 nM even after prolonged

exposure. In the presence of 100 nM TMC-114, virus strains had acquired Arg41Thr and Lys70Glu mutations, which were not concluded to be responsible for an apparent modest viral resistance to DRV.

However, when a mixture of multiple HIV-1 isolates resistant to multiple PIs was employed, highly DRV-resistant HIV-1 variants have been selected (Koh *et al.*, 2007). By 39 passages (in the presence of 1.0 μM DRV), the virus had acquired $\sim$170-fold IC_{50} increases than that against HIV-1_{NL4-3}. The virus relatively well propagated and was found to contain 12 mutations including Leu10Ile, Ile15Val, Lys20Arg, Leu24Ile, Val32Ile, Met36Ile, Met46Leu, Leu63Pro, Lys70Gln, Val82Ala, Ile84Val, and Leu89Met in the protease-encoding region. The virus at passage 30 with 0.3 μM DRV (HIV-$1_{DRV\text{-}P30}$) was titrated and examined for its susceptibility to DRV and other PIs using p24 assay. HIV-$1_{DRV\text{-}P30}$ was found highly resistant to DRV (110-fold greater IC_{50} than that against HIV-1_{NL4-3}) (Koh *et al.*, 2007). Altogether, these data suggest that DRV generally would not easily permit HIV-1 to develop significant resistance; however, HIV-1 could develop high levels of DRV-resistance, probably when superinfection with multi-PI-resistant HIV-1 variants and ensuing homologous recombinations occur.

Indeed, De Meyer *et al.* have found mutations including Val11Ile, Val32Ile, Leu33Phe, Ile47Val, Ile50Val, Ile54Leu/Met, Gly73Ser, Leu76Val, Ile84Val, and Leu89Val in HIV-1 strains isolated from those with a diminished virological response to DRV/r in the POWER studies (De Meyer, 2006). However, it is not known yet as to which amino acid mutations are major ones responsible for the apparent resistance to DRV.

E. Tipranavir and Darunavir

The approval of TPV as well as DRV (Fig. 2) for clinical use in drug-experienced patients demonstrated the success of structure-based drug design in the development of novel compounds. All previously clinically approved PIs were classified as peptidomimetics, due to the fact that they share structural similarity to the tetrahedral intermediate formed during hydrolytic cleavage of a peptide bond of the natural substrate (Randolph and DeGoey, 2004). In contrast, TPV was developed from a class of compounds known as dihydropyrones that are structurally similar to coumadin and were found to inhibit protease (Chrusciel and Strohbach, 2004). Traditional peptidomimetics all utilize a ubiquitous water molecule within the protease activity site in order to form hydrogen bonds with the flap domain of the enzyme. A key characteristic of TPV and other nonpeptidic PIs (NPPIs) in development is the absence of this water molecule as seen in crystal structures of NPPIs with protease. Instead, NPPIs contain a suitable chemical moiety that directly forms hydrogen bonds to the flap region without the need for water molecules. It is hypothesized that this allows

more entropically favorable interactions for the binding of these inhibitors to protease (Chrusciel and Strohbach, 2004).

The unique binding motif and structure of TPV has been thought to provide increased flexibility that would allow it to adjust to amino acid changes within the active site (Larder *et al.*, 2000; Turner *et al.*, 1998). Indeed, TPV exerts antiviral activity against a wide range of HIV variants resistant to multiple PIs (Back *et al.*, 2000; Larder *et al.*, 2000; Poppe *et al.*, 1997; Rusconi *et al.*, 2000). Nonetheless, resistance to TPV can occur although the acquisition of up to 10 mutations is required to achieve high levels of resistance (Doyon *et al.*, 2005). The mutations Leu33Phe and Ile84Val appear to be key substitutions responsible for the development of TPV resistance. Leu33Phe is a secondary mutation not present in the active site of protease and does not appear to affect TPV binding to protease. It has been suggested that this mutation protects the protease from autocatalysis (Schake, 2005). Ile84Val appears to directly diminish TPV binding by altering hydrophobic interactions that the drug has with this amino acid residue (Schake, 2004). Despite this, the potent activity of TPV against multi-PI-resistant HIV appears to stem from the hydrogen bond network it forms with the most invariant region of the protease active site that includes the catalytic Asp-25, as well as the backbone residues of Asp-29, Asp-30, Gly-48, and Ile-50 (Fig. 6) (Muzammil *et al.*, 2005; Schake, 2004).

At present, no randomized comparison data of TPV versus DRV are available, although there are a few reports on the benefits of TPV and DRV in those who have failed combination therapy with other PIs (Farthing *et al.*, 2006; Hill and Moyle, 2006). Such data show that both TPV and DRV are of useful option in patients who have failed other PIs; it is still to be determined how well they work against HIV-1 variants previously exposed to specific PI options. However, considering that TPV had been labeled a "last hope" PI option for people who have tried and failed other PIs, it appears that DRV—with its activity in patients no longer responding to TPV—certainly offers a significant advantage.

More profiles and the mechanisms of HIV-1 resistance to NPPIs such as TPV and DRV should be fully disclosed when more *in vitro* and clinical data involving more HIV-1 isolates and more individuals will continue to refine our knowledge of the molecular mechanisms of resistance, and provide us with novel insights for the prevention and treatment of drug-resistant HIV-1. A number of ongoing studies should further define the efficacy and safety of TPV and DRV in other patient populations and it is possible, in the near future, that these drugs could serve as a first-line therapeutic in HAART as well.

VIII. Conclusions

Successful antiviral drugs, in theory, exert their virus-specific effects without disturbing cellular metabolism or function. However, at present,

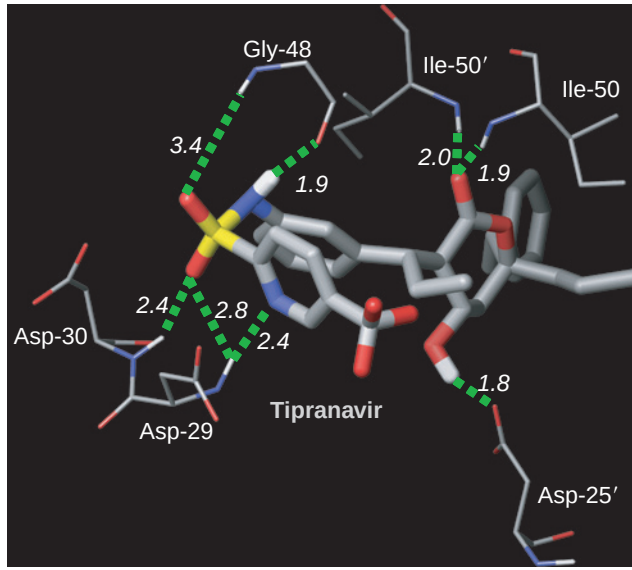


FIGURE 6 Hydrogen bond interactions of TPV with protease. Hydrogen bond interactions of TPV with Asp-29 and Asp-30 in the S2 subsite, with a catalytic aspartate (Asp-25′), and with flap residues Gly-48, Ile-50, and Ile-50′. The hydrogen bonds are shown in green dashed lines. The figure was generated using Maestro version 7.5.

no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives. Thus, the identification of new class of antiretroviral drugs which have a unique mechanism(s) of action and produce no or minimal side effects remains an important therapeutic objective.

A variety of novel anti-HIV-1 agents that target different steps in the HIV replication cycle are currently in preclinical trials and will undoubtedly improve our ability to manage HIV-1 infection when they are duly introduced into clinics. However, as has been the case with RTIs and PIs, the development of drug resistance will likely limit the effectiveness of these drugs as well. Thus, a key element in future drug design strategies will be to understand how drug resistance mutations affect the interaction of the drug with its target, and to develop compounds with the adaptability to inhibit these variants along with wild-type HIV-1. New generation RTIs and PIs have already shown promise in accomplishing this task, by utilizing knowledge of the molecular, biochemical, structural, and thermodynamic nature of drug resistance. This should serve as a model in the design of more effective anti-HIV-1 therapeutics.

Acknowledgments

This work was supported in part by the Intramural Research Program of Center for Cancer Research, National Cancer Institute, National Institutes of Health and in part by a Grant for Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan, and the Grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re emerging Infectious Diseases (Renkei Jigyo: No. 78, Kumamoto University) of Monbu-kagakusho (HM), and by a grant from the National Institutes of Health (GM53386, AKG).

References

Arasteh, K., Clumeck, N., Pozniak, A., Lazzarin, A., De Meyer, S., Muller, H., Peeters, M., Rinehart, A., and Lefebvre, E. (2005). TMC114/ritonavir substitution for protease inhibitor(s) in a non-suppressive antiretroviral regimen: A 14-day proof-of-principle trial. *AIDS* **19**(9), 943–947.

Back, N. K., van Wijk, A., Remmerswaal, D., van Monfort, M., Nijhuis, M., Schuurman, R., and Boucher, C. A. (2000). *In-vitro* tipranavir susceptibility of HIV-1 isolates with reduced susceptibility to other protease inhibitors. *AIDS* **14**(1), 101–102.

Carr, A. (2003). Toxicity of antiretroviral therapy and implications for drug development. *Nat. Rev. Drug Discov.* **2**(8), 624–634.

Chrusciel, R. A., and Strohbach, J. W. (2004). Non-peptidic HIV protease inhibitors. *Curr. Top. Med. Chem.* **4**(10), 1097–1114.

Collins, J. R., Burt, S. K., and Erickson, J. W. (1995). Flap opening in HIV-1 protease simulated by 'activated' molecular dynamics. *Nat. Struct. Biol.* **2**(4), 334–338.

Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., *et al.* (1995). *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374** (6522), 569–571.

Condra, J. H., Petropoulos, C. J., Ziermann, R., Schleif, W. A., Shivaprakash, M., and Emini, E. A. (2000). Drug resistance and predicted virologic responses to human immunodeficiency virus type 1 protease inhibitor therapy. *J. Infect. Dis.* **182**(3), 758–765.

Cote, H. C., Brumme, Z. L., and Harrigan, P. R. (2001). Human immunodeficiency virus type 1 protease cleavage site mutations associated with protease inhibitor cross-resistance selected by indinavir, ritonavir, and/or saquinavir. *J. Virol.* **75**(2), 589–594.

De Clercq, E. (2002). Strategies in the design of antiviral drugs. *Nat. Rev. Drug Discov.* **1**(1), 13–25.

De Meyer, S. (2006)."8th International Congress on Drug Therapy in HIV Infection, Glasgow, UK, 12–16 November 2006."

De Meyer, S., Azijn, H., Surleraux, D., Jochmans, D., Tahri, A., Pauwels, R., Wigerinck, P., and de Bethune, M. P. (2005). TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. *Antimicrob. Agents Chemother.* **49**(6), 2314–2321.

Deeks, S. G., Grant, R. M., Beatty, G. W., Horton, C., Detmer, J., and Eastman, S. (1998). Activity of a ritonavir plus saquinavir-containing regimen in patients with virologic evidence of indinavir or ritonavir failure. *AIDS* **12**(10), F97–F102.

Department of Health and Human Services (DHHS). (2006). "Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. October 10, 2006."

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., and Lamarre, D. (1996). Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J. Virol.* **70**(6), 3763–3769.

Doyon, L., Tremblay, S., Bourgon, L., Wardrop, E., and Cordingley, M. G. (2005). Selection and characterization of HIV-1 showing reduced susceptibility to the non-peptidic protease inhibitor tipranavir. *Antiviral Res.* **68**(1), 27–35.

Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X. C., Norbeck, D. W., Plattner, J. J., Rittenhouse, J. W., Turon, M., and Wideburg, N. (1990). Design, activity, and 2.8 Å crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. *Science* **249**, 527–533.

Eron, J., Jr., Yeni, P., Gathe, J., Jr., Estrada, V., DeJesus, E., Staszewski, S., Lackey, P., Katlama, C., Young, B., Yau, L., Sutherland-Phillips, D., Wannamaker, P., *et al.* (2006). The KLEAN study of fosamprenavir-ritonavir versus lopinavir-ritonavir, each in combination with abacavir-lamivudine, for initial treatment of HIV infection over 48 weeks: A randomised non-inferiority trial. *Lancet* **368**(9534), 476–482.

Farthing, C., Ward, D., and Hicks, C. (2006). Tipranavir/r demonstrates superior and durable treatment responses compared with comparator PI/r in highly treatment experienced patients: Week 96 RESIST 1 and 2 results [Abstract 1385]. *In* "46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006."

Fitzgerald, P. M., and Springer, J. P. (1991). Structure and function of retroviral proteases. *Annu. Rev. Biophys. Biophys. Chem.* **20**, 299–320.

Fumero, E., and Podzamczer, D. (2003). New patterns of HIV-1 resistance during HAART. *Clin. Microbiol. Infect.* **9**(11), 1077–1084.

Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M. F., Nagashima, K., Gorelick, R. J., Mardy, S., Tang, C., Summers, M. F., and Mitsuya, H. (2002). Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. *J. Biol. Chem.* **277**(8), 5952–5961.

Gatanaga, H., Das, D., Suzuki, Y., Yeh, D. D., Hussain, K. A., Ghosh, A. K., and Mitsuya, H. (2006). Altered HIV-1 Gag protein interactions with cyclophilin A (CypA) on the acquisition of H219Q and H219P substitutions in the CypA binding loop. *J. Biol. Chem.* **281**(2), 1241–1250.

Ghosh, A. K., Thompson, W. J., McKee, S. P., Duong, T. T., Lyle, T. A., Chen, J. C., Darke, P. L., Zugay, J., Emini, E. A., Schleif, W. A., Huff, J. R., and Anderson, P. S. (1993). 3-Tetrahydrofuran and pyranyl urethanes as high affinity P_2-ligands for HIV-1 protease inhibitors. *J. Med. Chem.* **36**, 292–294.

Ghosh, A. K., Thompson, W. J., Fitzgerald, P. M., Culberson, J. C., Axel, M. G., McKee, S. P., Huff, J. R., and Anderson, P. S. (1994). Structure-based design of HIV-1 protease inhibitors: Replacement of two amides and a 10 pi-aromatic system by a fused bis-tetrahydrofuran. *J. Med. Chem.* **37**(16), 2506–2508.

Ghosh, A. K., Kincaid, J. F., Walters, D. E., Chen, Y., Chaudhuri, N. C., Thompson, W. J., Culberson, C., Fitzgerald, P. M., Lee, H. Y., McKee, S. P., Munson, P. M., Duong, T. T., *et al.* (1996). Nonpeptidal P2 ligands for HIV protease inhibitors: Structure-based design, synthesis, and biological evaluation. *J. Med. Chem.* **39**(17), 3278–3290.

Ghosh, A. K., Krishnan, K., Walters, D. E., Cho, W., Cho, H., Koo, Y., Trevino, J., Holland, L., and Buthod, J. (1998). Structure based design: Novel spirocyclic ethers as nonpeptidal P2-ligands for HIV protease inhibitors. *Bioorg. Med. Chem. Lett.* **8**(8), 979–982.

Ghosh, A. K., Shin, D., Swanson, L., Krishnan, K., Cho, H., Hussain, K. A., Walters, D. E., Holland, L., and Buthod, J. (2001). Structure-based design of nonpeptide HIV protease inhibitors. *IL Farmaco* **56**, 29–35.

Ghosh, A. K., Pretzer, E., Cho, H., Hussain, K. A., and Duzgunes, N. (2002). Antiviral activity of UIC-PI, a novel inhibitor of the human immunodeficiency virus type 1 protease. *Antiviral Res.* **54**, 29–36.

Ghosh, A. K., Swanson, L. M., Cho, H., Leshchenko, S., Hussain, K. A., Kay, S., Walters, D. E., Koh, Y., and Mitsuya, H. (2005). Structure-based design: Synthesis and biological evaluation of a series of novel cycloamide-derived HIV-1 protease inhibitors. *J. Med. Chem.* **48**(10), 3576–3585.

Ghosh, A. K., Schiltz, G., Perali, R. S., Leshchenko, S., Kay, S., Walters, D. E., Koh, Y., Maeda, K., and Mitsuya, H. (2006a). Design and synthesis of novel HIV-1 protease inhibitors incorporating oxyindoles as the P2′-ligands. *Bioorg. Med. Chem. Lett.* **16**(7), 1869–1873.

Ghosh, A. K., Sridhar, P. R., Leshchenko, S., Hussain, A. K., Li, J., Kovalevsky, A. Y., Walters, D. E., Wedekind, J. E., Grum-Tokars, V., Das, D., Koh, Y., Maeda, K., *et al.* (2006b). Structure-based design of novel HIV-1 protease inhibitors to combat drug resistance. *J. Med. Chem.* **49** (17), 5252–5261.

Ghosh, A. K., Sridhar, P., Kumaragurubaran, N., Koh, Y., Weber, I. T., and Mitsuya, H. (2006c). Bis-tetrahydrofuran: A privileged ligand for darunavir and a new generation of HIV-protease inhibitors that combat drug-resistance. *Chem. Med. Chem.* **1**, 937–950.

Gisolf, E. H., van Heeswijk, R. P., Hoetelmans, R. W., and Danner, S. A. (2000). Decreased exposure to saquinavir in HIV-1-infected patients after long-term antiretroviral therapy including ritonavir and saquinavir. *AIDS* **14**(7), 801–805.

Grabar, S., Weiss, L., and Costagliola, D. (2006). HIV infection in older patients in the HAART era. *J. Antimicrob. Chemother.* **57**(1), 4–7.

Hertogs, K., Bloor, S., Kemp, S. D., Van den Eynde, C., Alcorn, T. M., Pauwels, R., Van Houtte, M., Staszewski, S., Miller, V., and Larder, B. A. (2000). Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: A survey of over 6000 samples. *AIDS* **14**(9), 1203–1210.

Hicks, C. B., Cahn, P., Cooper, D. A., Walmsley, S. L., Katlama, C., Clotet, B., Lazzarin, A., Johnson, M. A., Neubacher, D., Mayers, D., and Valdez, H. (2006). Durable efficacy of tipranavir-ritonavir in combination with an optimised background regimen of antiretroviral drugs for treatment-experienced HIV-1-infected patients at 48 weeks in the Randomized Evaluation of Strategic Intervention in multi-drug reSistant patients with Tipranavir (RESIST) studies: An analysis of combined data from two randomised open-label trials. *Lancet* **368**(9534), 466–475.

Hill, A., and Moyle, G. (2006). Relative week 48 antiviral efficacy of TMC114/r and tipranavir/r versus control PI in the POWER and RESIST trials [Abstract H-1386]. *In* "46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006."

Hirsch, H. H., Kaufmann, G., Sendi, P., and Battegay, M. (2004). Immune reconstitution in HIV-infected patients. *Clin. Infect. Dis.* **38**(8), 1159–1166.

Ho, D. D., Toyoshima, T., Mo, H., Kempf, D. J., Norbeck, D., Chen, C. M., Wideburg, N. E., Burt, S. K., Erickson, J. W., and Singh, M. K. (1994). Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. *J. Virol.* **68**(3), 2016–2020.

Hong, L., Hartsuck, J. A., Foundling, S., Ermolieff, J., and Tang, J. (1998). Active-site mobility in human immunodeficiency virus, type 1, protease as demonstrated by crystal structure of A28S mutant. *Protein Sci.* **7**(2), 300–305.

Hornak, V., Okur, A., Rizzo, R. C., and Simmerling, C. (2006). HIV-1 protease flaps spontaneously open and reclose in molecular dynamics simulations. *Proc. Natl. Acad. Sci. USA* **103**(4), 915–920.

Jacobsen, H., Ahlborn-Laake, L., Gugel, R., and Mous, J. (1992). Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. *J. Virol.* **66**, 5087–5091.

Johnson, M. A., Gathe, J. C., Jr., Podzamczer, D., Molina, J. M., Naylor, C. T., Chiu, Y. L., King, M. S., Podsadecki, T. J., Hanna, G. J., and Brun, S. C. (2006). A once-daily lopinavir/ritonavir-based regimen provides noninferior antiviral activity compared with a twice-daily regimen. *J. Acquir. Immune Defic. Syndr.* **43**(2), 153–160.

Kaplan, A. H., Michael, S. F., Wehbie, R. S., Knigge, M. F., Paul, D. A., Everitt, L., Kempf, D. J., Norbeck, D. W., Erickson, J. W., and Swanstrom, R. (1994). Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased

sensitivity to an inhibitor of the viral protease. *Proc. Natl. Acad. Sci. USA* **91**(12), 5597–5601.

Katlama, C., Esposito, R., Gatell, J. M., Goffard, J. C., Grinsztejn, B., Pozniak, A., Rockstroh, J., Stoehr, A., Vetter, N., Yeni, P., Parys, W., and Vangeneugden, T. (2007). Efficacy and safety of TMC114/ritonavir in treatment-experienced HIV patients: 24-week results of POWER 1. *AIDS* **21**(4), 395–402.

Kavlick, M. F., and Mitsuya, H. (2001). The emergence of drug resistant HIV-1 variants and its impact on antiretroviral therapy of HIV-1 infection. *In* "The Art of Antiretroviral Therapy" (E. D. Clercq, Ed.). American Society for Microbiology, Washington, DC.

Kemper, C. A., Witt, M. D., Keiser, P. H., Dube, M. P., Forthal, D. N., Leibowitz, M., Smith, D. S., Rigby, A., Hellmann, N. S., Lie, Y. S., Leedom, J., Richman, D., *et al.* (2001). Sequencing of protease inhibitor therapy: Insights from an analysis of HIV phenotypic resistance in patients failing protease inhibitors. *AIDS* **15**(5), 609–615.

Kempf, D., Norbeck, D., Codacovi, L., Wang, X., Kohlbrenner, W., Wideburg, N., Paul, D., Knigge, M., Vasavanonda, S., Craig-Kennard, A., Saldivar, A., Rosenbrook, W., Jr., *et al.* (1990). Structure-based, C_2 symmetric inhibitors of HIV protease. *J. Med. Chem.* **33**, 2687–2689.

Kempf, D. J., Marsh, K. C., Kumar, G., Rodrigues, A. D., Denissen, J. F., McDonald, E., Kukulka, M. J., Hsu, A., Granneman, G. R., Baroldi, P. A., Sun, E., Pizzuti, D., *et al.* (1997). Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. *Antimicrob. Agents Chemother.* **41**(3), 654–660.

Kim, E. E., Baker, C. T., Dwyer, M. D., Murcko, M. A., Rao, B. G., Tung, R. D., and Navia, M. A. (1995). Crystal structure of HIV-1 protease in complex with VX-478, a potent and orally bioavailable inhibitor of the enzyme. *J. Am. Chem. Soc.* **117**, 1181–1182.

King, N. M., Prabu-Jeyabalan, M., Nalivaika, E. A., and Schiffer, C. A. (2004a). Combating susceptibility to drug resistance: Lessons from HIV-1 protease. *Chem. Biol.* **11**(10), 1333–1338.

King, N. M., Prabu-Jeyabalan, M., Nalivaika, E. A., Wigerinck, P., de Bethune, M. P., and Schiffer, C. A. (2004b). Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. *J. Virol.* **78** (21), 12012–12021.

Koh, Y., Nakata, H., Maeda, K., Ogata, H., Bilcer, G., Devasamudram, T., Kincaid, J. F., Boross, P., Wang, Y. F., Tie, Y., Volarath, P., Gaddis, L., *et al.* (2003). Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **47**, 3123–3129.

Koh, Y., Towata, T., Ghosh, A., and Mitsuya, H. (2007). *In* "14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, February 25–28, 2007."

Larder, B. A., Hertogs, K., Bloor, S., van den Eynde, C. H., DeCian, W., Wang, Y., Freimuth, W. W., and Tarpley, G. (2000). Tipranavir inhibits broadly protease inhibitor-resistant HIV-1 clinical samples. *AIDS* **14**, 1943–1948.

Lin, Y., Lin, X., Hong, L., Foundling, S., Heinrikson, R. L., Thaisrivongs, S., Leelamanit, W., Raterman, D., Shah, M., Dunn, B. M., and Tang, J. (1995). Effect of point mutations on the kinetics and the inhibition of human immunodeficiency virus type 1 protease: Relationship to drug resistance. *Biochemistry* **34**(4), 1143–1152.

Little, S. J., Holte, S., Routy, J. P., Daar, E. S., Markowitz, M., Collier, A. C., Koup, R. A., Mellors, J. W., Connick, E., Conway, B., Kilby, M., Wang, L., *et al.* (2002). Antiretroviral-drug resistance among patients recently infected with HIV. *N. Engl. J. Med.* **347**(6), 385–394.

Logsdon, B. C., Vickrey, J. F., Martin, P., Proteasa, G., Koepke, J. I., Terlecky, S. R., Wawrzak, Z., Winters, M. A., Merigan, T. C., and Kovari, L. C. (2004). Crystal structures of a multidrug-

resistant human immunodeficiency virus type 1 protease reveal an expanded active-site cavity. *J. Virol.* **78**(6), 3123–3132.

Markowitz, M., Mo, H., Kempf, D. J., Norbeck, D. W., Bhat, T. N., Erickson, J. W., and Ho, D. D. (1995). Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. *J. Virol.* **69**, 701–706.

Mitsuya, H., and Broder, S. (1986). Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy virus-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**, 1911–1915.

Mitsuya, H., and Broder, S. (1987). Strategies for antiviral therapy in AIDS. *Nature* **325**, 773–778.

Mitsuya, H., and Erickson, J. (1999). Discovery and development of antiretroviral therapeutics for HIV infection. *In* "Textbook of AIDS Medicine" (T. C. Merigan, J. G. Bartlet, and D. Bolognesi, Eds.), pp. 751–780. Williams & Wilkins, Baltimore.

Mitsuya, H., Weinhold, K. J., Furman, P. A., St Clair, M. H., Nusinoff Lehrman, S., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985). 3′-Azido-3′-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**, 7096–7100.

Mitsuya, H., Yarchoan, R., and Broder, S. (1990). Molecular targets for AIDS therapy. *Science* **249**, 1533–1544.

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., *et al.* (1996). Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat. Med.* **2**(7), 760–766.

Muzammil, S., Kang, L. W., Armstrong, A. A., Jakalian, A., Bonneau, P. R., Schmelmer, V., Amzel, L. M., and Freire, E. (2005). Unique response of tipranavir to multi-drug resistant HIV-1 protease suggests new ways of combating drug resistance. *Antiviral Res.* **10**(S70), Abstract 63.

Ohtaka, H., and Freire, E. (2005). Adaptive inhibitors of the HIV-1 protease. *Prog. Biophys. Mol. Biol.* **88**(2), 193–208.

Otto, M. J., Garber, S., Winslow, D. L., Reid, C. D., Aldrich, P., Jadhav, P. K., Patterson, C. E., Hodge, C. N., and Cheng, Y. S. (1993). *In vitro* isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease. *Proc. Natl. Acad. Sci. USA* **90**(16), 7543–7547.

Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006). How many drug targets are there? *Nat. Rev. Drug Discov.* **5**, 993–996.

Peters, S., Munoz, M., Yerly, S., Sanchez-Merino, V., Lopez-Galindez, C., Perrin, L., Larder, B., Cmarko, D., Fakan, S., Meylan, P., and Telenti, A. (2001). Resistance to nucleoside analog reverse transcriptase inhibitors mediated by human immunodeficiency virus type 1 p6 protein. *J. Virol.* **75**(20), 9644–9653.

Poppe, S. M., Slade, D. E., Chong, K. T., Hinshaw, R. R., Pagano, P. J., Markowitz, M., Ho, D. D., Mo, H., Gorman, R. R., 3rd, Dueweke, T. J., Thaisrivongs, S., and Tarpley, W. G. (1997). Antiviral activity of the dihydropyrone PNU-140690, a new nonpeptidic human immunodeficiency virus protease inhibitor. *Antimicrob. Agents Chemother.* **41**(5), 1058–1063.

Prabu-Jeyabalan, M., Nalivaika, E., and Schiffer, C. A. (2000). How does a symmetric dimer recognize an asymmetric substrate? A substrate complex of HIV-1 protease. *J. Mol. Biol.* **301**(5), 1207–1220.

Prabu-Jeyabalan, M., Nalivaika, E., and Schiffer, C. A. (2002). Substrate shape determines specificity of recognition for HIV-1 protease: Analysis of crystal structures of six substrate complexes. *Structure* **10**(3), 369–381.

Prabu-Jeyabalan, M., Nalivaika, E. A., King, N. M., and Schiffer, C. A. (2003). Viability of a drug-resistant human immunodeficiency virus type 1 protease variant: Structural insights for better antiviral therapy. *J. Virol.* **77**(2), 1306–1315.

Randolph, J. T., and DeGoey, D. A. (2004). Peptidomimetic inhibitors of HIV protease. *Curr. Top. Med. Chem.* **4**(10), 1079–1095.

Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Duncan, I. B., Galpin, S. A., Handa, B. K., Kay, J., and Krohn, A. (1990). Rational design of peptide-based HIV proteinase inhibitors. *Science* **248**, 358–361.

Rusconi, S., La Seta Catamancio, S., Citterio, P., Kurtagic, S., Violin, M., Balotta, C., Moroni, M., Galli, M., and d'Arminio-Monforte, A. (2000). Susceptibility to PNU-140690 (Tipranavir) of human immunodeficiency virus type 1 isolates derived from patients with multidrug resistance to other protease inhibitors. *Antimicrob. Agents Chemother.* **44**(5), 1328–1332.

Schake, D. (2004). How flexible is tipranavir in complex with the HIV-1 protease active site? *AIDS* **18**(3), 579–580.

Schake, D. (2005). Molecular significance of tipranavir related codon 33 protease gene mutations. *AIDS* **19**(2), 218–219.

Siliciano, J. D., Siliciano, R. F., Blankson, J. N., Persaud, D., Siliciano, R. F., Pierson, T., McArthur, J., Siliciano, R. F., and Siliciano, R. (2004). A long-term latent reservoir for HIV-1: Discovery and clinical implications. *J. Antimicrob. Chemother.* **54**(1), 6–9.

Simon, V., and Ho, D. D. (2003). HIV-1 dynamics *in vivo*: Implications for therapy. *Nat. Rev. Microbiol.* **1**(3), 181–190.

Sommadossi, J. P. (1999). HIV protease inhibitors: Pharmacologic and metabolic distinctions. *AIDS* **13**(Suppl. 1), S29–S40.

Surleraux, D. L., de Kock, H. A., Verschueren, W. G., Pille, G. M., Maes, L. J., Peeters, A., Vendeville, S., De Meyer, S., Azijn, H., Pauwels, R., de Bethune, M. P., King, N. M., *et al.* (2005a). Design of HIV-1 protease inhibitors active on multidrug-resistant virus. *J. Med. Chem.* **48**(6), 1965–1973.

Surleraux, D. L., Tahri, A., Verschueren, W. G., Pille, G. M., de Kock, H. A., Jonckers, T. H., Peeters, A., De Meyer, S., Azijn, H., Pauwels, R., de Bethune, M. P., King, N. M., *et al.* (2005b). Discovery and selection of TMC114, a next generation HIV-1 protease inhibitor. *J. Med. Chem.* **48**(6), 1813–1822.

Tamiya, S., Mardy, S., Kavlick, M. F., Yoshimura, K., and Mitsuya, H. (2004). Amino acid insertions near Gag cleavage sites restore the otherwise compromised replication of human immunodeficiency virus type 1 variants resistant to protease inhibitors. *J. Virol.* **78**(21), 12030–12040.

Tanaka, M., Srinivas, R. V., Ueno, T., Kavlick, M. F., Hui, F. K., Fridland, A., Driscoll, J. S., and Mitsuya, H. (1997). *In vitro* induction of human immunodeficiency virus type 1 variants resistant to 2′-beta-Fluoro-2′,3′-dideoxyadenosine. *Antimicrob. Agents Chemother.* **41**(6), 1313–1318.

Tie, Y., Boross, P. I., Wang, Y. F., Gaddis, L., Hussain, A. K., Leshchenko, S., Ghosh, A. K., Louis, J. M., Harrison, R. W., and Weber, I. T. (2004). High resolution crystal structures of HIV-1 protease with a potent non-peptide inhibitor (UIC-94017) active against multi-drug-resistant clinical strains. *J. Mol. Biol.* **338**(2), 341–352.

Tisdale, M., Myers, R. E., Maschera, B., Parry, N. R., Oliver, N. M., and Blair, E. D. (1995). Cross-resistance analysis of human immunodeficiency virus type 1 variants individually selected for resistance to five different protease inhibitors. *Antimicrob. Agents Chemother.* **39**(8), 1704–1710.

Turk, B. (2006). Targeting proteases: Successes, failures and future prospects. *Nat. Rev. Drug Discov.* **5**, 785–799.

Turner, S. R., Strohbach, J. W., Tommasi, R. A., Aristoff, P. A., Johnson, P. D., Skulnick, H. I., Dolak, L. A., Seest, E. P., Tomich, P. K., Bohanon, M. J., Horng, M. M., Lynn, J. C., *et al.* (1998). Tipranavir (PNU-140690): A potent, orally bioavailable nonpeptidic HIV

protease inhibitor of the 5,6-dihydro-4-hydroxy-2-pyrone sulfonamide class. *J. Med. Chem.* **41**(18), 3467–3476.

Ward, D., Lalezari, J., Thompson, M., Dejesus, E., Lamarca, A., Hawkins, T., Green, S., Myers, R., Ford, S., Ng-Cashin, J., Stclair, M., Reddy, S., *et al.* (2005). Preliminary Antiviral Activity and Safety of 640385/Ritonavir in HIV-infected Patients (Study HPR10006); an 8-Week Interim Analysis. *In* "45th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington DC.", Abstract H-412.

Watkins, T., Resch, W., Irlbeck, D., and Swanstrom, R. (2003). Selection of high-level resistance to human immunodeficiency virus type 1 protease inhibitors. *Antimicrob. Agents Chemother.* **47**(2), 759–769.

Winters, M. A., Coolley, K. L., Girard, Y. A., Levee, D. J., Hamdan, H., Shafer, R. W., Katzenstein, D. A., and Merigan, T. C. (1998). A 6-basepair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. *J. Clin. Invest.* **102**(10), 1769–1775.

Yarchoan, R., Mitsuya, H., Thomas, R. V., Pluda, J. M., Hartman, N. R., Perno, C. F., Marczyk, K. S., Allain, J. P., Johns, D. G., and Broder, S. (1989a). *In vivo* activity against HIV and favorable toxicity profile of 2′,3′-dideoxyinosine. *Science* **245**, 412–415.

Yarchoan, R., Mitsuya, H., Myers, C. E., and Broder, S. (1989b). Clinical pharmacology of 3′-azido-2′,3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. *N. Engl. J. Med.* **321**, 726–738.

Yarchoan, R., Lietzau, J. A., Nguyen, B. Y., Brawley, O. W., Pluda, J. M., Saville, M. W., Wyvill, K. M., Steinberg, S. M., Agbaria, R., Mitsuya, H., and Broder, S. (1994). A randomized pilot study of alternating or simultaneous zidovudine and didanosine therapy in patients with symptomatic human immunodeficiency virus infection. *J. Infect. Dis.* **169** (1), 9–17.

Yates, P., Hazen, R., St Clair, M., Boone, L., and Elston, R. (2004). *In vitro* selection and characterisation of resistance to the new HIV protease inhibitor GW640385. *Antivir. Ther.* **9**(S16), 12.

Yin, P. D., Das, D., and Mitsuya, H. (2006). Overcoming HIV drug resistance through rational drug design based on molecular, biochemical, and structural profiles of HIV resistance. *Cell. Mol. Life Sci.* **63**, 1706–1724.

Yoshimura, K., Kato, R., Kavlick, M. F., Nguyen, A., Maroun, V., Maeda, K., Hussain, K. A., Ghosh, A. K., Gulnik, S. V., Erickson, J. W., and Mitsuya, H. (2002). A potent human immunodeficiency virus type 1 protease inhibitor, UIC-94003 (TMC-126), and selection of a novel (A28S) mutation in the protease active site. *J. Virol.* **76**(3), 1349–1358.

Yusa, K., and Harada, S. (2004). Acquisition of multi-PI (protease inhibitor) resistance in HIV-1 *in vivo* and *in vitro*. *Curr. Pharm. Des.* **10**(32), 4055–4064.

Zhang, Y. M., Imamichi, H., Imamichi, T., Lane, H. C., Falloon, J., Vasudevachari, M. B., and Salzman, N. P. (1997). Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. *J. Virol.* **71**(9), 6662–6670.

This page intentionally left blank

Elena A. Semenova, Christophe Marchand, and Yves Pommier

Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892–4255

HIV-1 Integrase Inhibitors: Update and Perspectives

I. Chapter Overview

HIV replication requires the insertion of the viral genome inside the nuclear genome of infected cells through a recombination process catalyzed by the virus-encoded enzyme, integrase (IN). HIV IN has recently been recognized as a reachable antiviral target following the promising results of IN inhibitors in clinical trials. The present chapter focuses on the recent advances in understanding the cellular mechanisms of HIV integration and the sites of actions of inhibitors. It also provides an extensive list of the known mutations that have been characterized for HIV-1 IN with their impact on IN activity, viral replication, and response to anti-IN drugs.

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56007-2

Novel rational approaches for inhibiting HIV integration are also discussed, as well as the two IN inhibitors in clinical trials and other selected inhibitors in development.

II. Foreword

The encouraging results reported for two IN inhibitors in clinical trials and the recent insights in the cellular cofactors of DNA integration have renewed interest in HIV IN pharmacology and cellular biology. The current chapter gives an overview of the functions of IN and the cofactors for integration. It focuses on pharmacological approaches to interfere with integration and provides an extensive list of IN mutations with their functional and pharmacological impacts. References have been kept to a minimum. Further information can be found in recent reviews (Dayam *et al.*, 2006; Marchand *et al.*, 2006a; Pommier *et al.*, 2005; Savarino, 2006; Semenova *et al.*, 2006b; Zhao *et al.*, 2007).

III. Integration: A Crucial Step in the HIV Life Cycle

Like other retroviruses, the HIV genome consists of single-stranded RNA. During infection, the viral RNA is released into the host cell following fusion of the viral particles to the cell membrane (Fig. 1). The viral RNA then serves as a template for the synthesis of a double-stranded DNA copy of the viral RNA (cDNA) bearing long terminal repeats (LTRs) by the HIV-encoded reverse transcriptase (Sierra *et al.*, 2005). The conversion of the viral RNA into cDNA is necessary for making new viral RNA copies and for transcribing the virally encoding genes. Transcription of the viral cDNA also requires its insertion into a host chromosome. That insertion (integration) is catalyzed by the HIV-encoded enzyme—IN. The viral cDNA integrated into a host chromosome is called provirus (Fig. 1). Depending on the sites of integration, the provirus can be constitutively transcribed if it is integrated near an active promoter, or remain silent until a stress response triggers transcription. Transcription of the viral genome and of the viral genes followed by translation, packaging, fusion, and maturation supply the molecular components for the release of the new infectious viral particles (Fig. 1).

A. HIV-1 IN Structure

The three viral enzymes (protease, reverse transcriptase, and IN) are encoded within the HIV *pol* gene and translated as a polyprotein (Fig. 2). IN (32 kDa) is released from the polyprotein by the HIV protease during

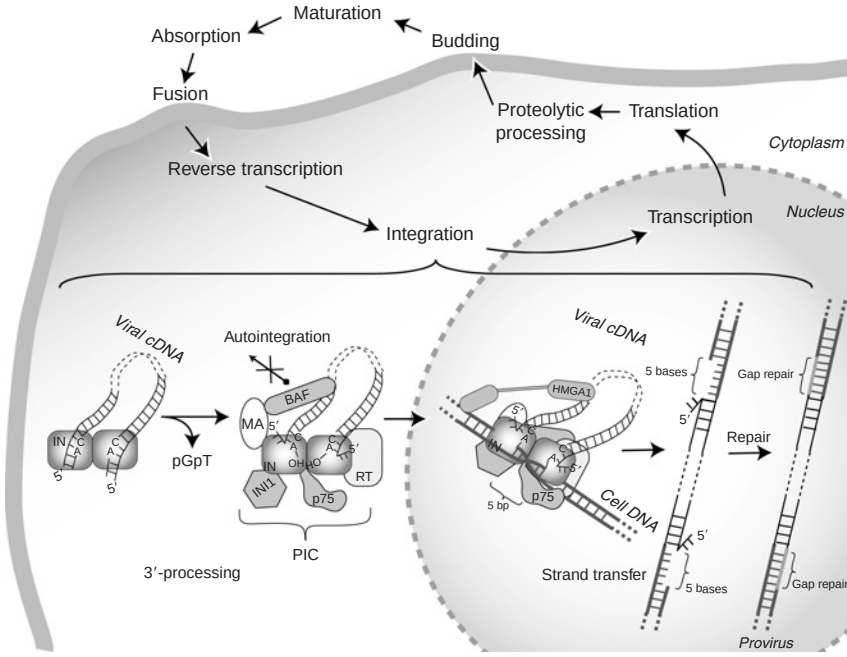


FIGURE 1 Integration in the HIV life cycle. IN: integrase, PIC: preintegration complex, BAF: barrier-to-autointegration factor, MA: HIV matrix protein, INI1: IN interactor 1, LEDGF/p75: lens epithelium-derived growth factor/transcription coactivator p75, RT: HIV reverse transcriptase, and HMGA1: high mobility group chromosomal protein A1.

maturation. The IN protein consists of three domains: N-terminal, core (or catalytic), and C-terminal domains (Fig. 2; Chiu and Davies, 2004). The N-terminal domain enhances IN multimerization through zinc coordination (HHCC motif; See Fig. 2) and promotes concerted integration of the two viral cDNA ends together into a host cell chromosome. The C-terminal domain is responsible for metal-independent, sequence-independent DNA binding. Each HIV-1 IN molecule contains a catalytic site within the core domain bearing three essential amino acids: Asp64, Asp116, and Glu152 (D,D-35-E motif). These acidic residues coordinate at least one and probably two divalent cations (Mg^{2+} or Mn^{2+}) that form a bridge with the DNA substrates (see Fig. 3; Marchand *et al.*, 2006a). Mutation of any of these residues abolishes IN enzymatic activities and viral replication (Table I). IN functions as a multimer.

B. Chemistry of Retroviral Integration

During the first reaction catalyzed by IN [3′-processing (3′-P)], the donor (viral) DNA is hydrolyzed immediately 3′ from the conserved CA

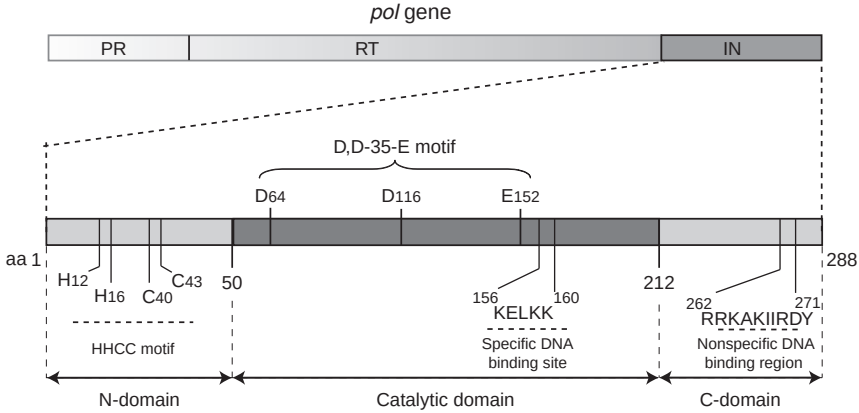


FIGURE 2 Functional domains of HIV-1 integrase. IN: integrase, RT: HIV reverse transcriptase, PR: HIV protease.

dinucleotide at both 3′ ends of the LTRs (Vink *et al.*, 1991; Fig. 3a). 3′-P releases 3′-terminal nucleotides (generally pGpT dinucleotide for HIV-1) and generates 3′-hydroxyl nucleophilic ends at both ends of the viral DNA. The next step, integration [strand transfer (ST)] proceeds in the nucleus through a transesterification reaction, where the processed nucleophilic 3′-OH ends of the donor (viral) cDNA are inserted into the backbone of the target (host) DNA (Vink *et al.*, 1990; Fig. 3b and c). Both ends insert with a 5-bp stagger across the DNA major groove of the target chromosomal DNA following the trimming of the integrated HIV cDNA junctions, gap filling, and ligation, which are probably carried out by the host cell DNA repair mechanism (Fig. 1; Pommier *et al.*, 2005).

C. Integration Occurs Within a Large Macromolecular Complex

Cellular integration requires several cofactors in addition to IN (Van Maele *et al.*, 2006). The preintegration complex (PIC) is a crucial structural unit required for integration. The PIC contains proteins from both the viral core (matrix, nucleocapsid, reverse transcriptase) and the host cell [lens epithelium-derived growth factor (LEDGF/p75), integrase interactor 1 (INI1), barrier-to-autointegration factor (BAF), high mobility group chromosomal protein A1 (HMGA1)] (Fig. 1). The viral cDNA is probably bound to IN immediately following reverse transcription. IN also binds directly to LEDGF/p75, INI1, reverse transcriptase, and matrix (Van Maele and Debyser, 2005).

Despite many reports describing the importance of cofactors for HIV integration, our understanding of the mechanisms regulating integration

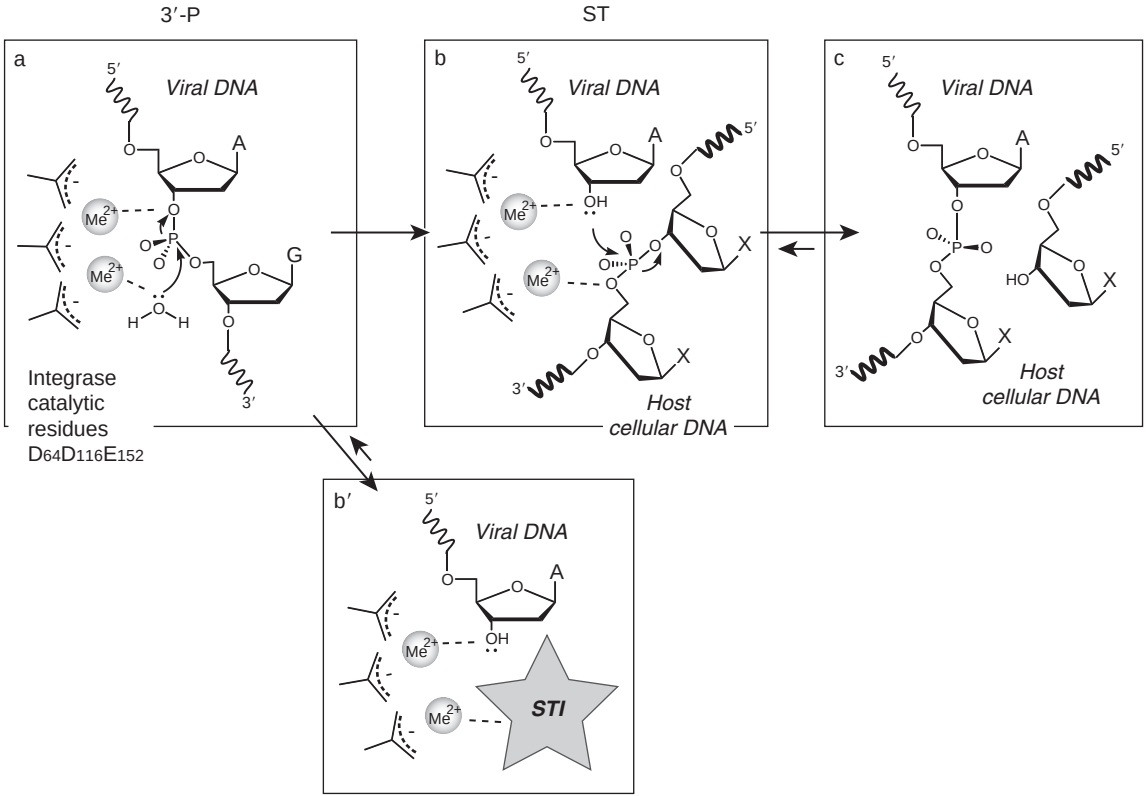


FIGURE 3 Biochemical steps of retroviral integration and proposed binding of strand transfer inhibitors (STI) at the interface of the integrase (IN)–viral DNA complex. (a) IN binds to the LTR viral DNA (thin wavy line) and catalyzes the nucleophilic attack of the phosphodiester viral DNA backbone 3′ from a conserved CA dinucleotide by a water molecule (3′-P: 3′-processing). (b) Following translocation to the nucleus, IN promotes the nucleophilic attack of the host cellular DNA (thick wavy line) by the 3′-hydroxy viral nucleophilic end, which results in strand transfer (ST) and cleavage of the host DNA (c). (b′) STI bind to the IN–viral DNA complex following 3′-P and probably interfere with the binding of host cellular DNA.

TABLE 1 Catalytic activities of HIV-1 IN mutants[a]

Mutations	*3′-P*	*ST*	*Dis*	*Inf*	*Interaction (References)*[b]
H12A;N*	+	+	+++	− (−)	PIC (1–4)
H12C*			+++	(−)	(5,6)
H12N/H16N	−	−	+		(2)
H12C/H16C			+++		(5)
H12Q/H16Y				(−)	(7)
Y15A				(−)	(8)
H16A*				−	(3)
H16C;V*	+++	+++	+++	(−)	(5,6)
K34A	++	++		− (−)	PIC (4)
K46A				+++	(4)
C40A;S*	−	−	+	(−)	(1,9)
C40S/C43S	−	−	++		(1)
D41A/K42A				+	(3)
C43A;S;L*	+	+		(−)	(7,9,10)
M50A				+	(9,10)
H51A*				+	(3)
H51A/D55V				−	(3)
Q53C	+++	+++	++	(−)	(5,6)
Q53K				+++	(11)
D55A				−	(3)
D55A;S				+++	(11)
D55K				−	(11)
C56A*				+++	(12)
C56S*	+++	+++		+++	(13,14)
C56S/C65S				Del	(13)
C56S/C130S				−	(13)
C56S/C65S/C280S	++	++	+++		(13,15)
C56S/C65S/C130S/ C280S	++	−	+++		(13)
C56S/C65S/Q148C/ C280S	++	−			(13,15)
Q62E	++	++	+++		(16)
Q62K				−	(11)
Q62N	+	−	−		(17)
D64C;R*			−		(17)
D64A;E;N;V*	−	−	−	−	(1,3,5,6,9, 18,19)
D64A/D116A				−	(3)
D64R/D116R			−		(17)
D64A/E152A				−	(3)
D64A/D116A/ E152A				−	(3)
C65S*	+++	+++		+++	(13,14)
C65A*				+++	(12)
C65S/C130S				−	(13)
T66A*	+	++	++	(++)	(17,20)
T66I*	++/+++	++/+++	++	+/++	L-708,906; L-731,988; S-1360; 118D-24; L-CA (21–27)
T66I/L74M	+	+		+	L-708,906; S-1360; (23)

T66I/S153Y				++	L-708,906; L-731,988; 118D-24; L-CA (24–26)	T115A;S	+++	+++	+++	+++	(1,9,18,31,32)
						D116A;E;I;N*	–	–	–	– (–)	(1,5,6,9,17, 18, 31–34)
						D116C*			+		(17)
						D116A/E152A**				–	(3)
T66I/M154I	++	++	++	+	L-731,988; L-CA (25)	D116/A23V**	–	++	–		(35)
						D116/E11D	–	++	–		(35)
T66I/L74M/S230R	+	+		++	S-1360; L-708, 906 (23)	D116/L28R/C65S/ T210N/L213I	–	+	–		(35)
						N117K;Q	++	++	++	Del	(1,11,17,32,33)
H67E*				Del	(11)	N117S	+	+	+		(17)
H67S*	+++	+++	++		(17)	G118A	+++		+++	+++	(32,33)
H67Q/K71E				–	(11)	S119T;G;A;K	+++	+++			(29)
E69A/K71A				–	(3)	N120Q;S*	+++	+++	+++		(17)
K71E*				+++	(11)	N120I;L;E;G*				(–)	(6)
V72I					L-870,810 (26)	N120L;K*				Del	(11)
L74M*	+++	+++			L-708,906; S-1360 (23,27)	N120L/Q148K				–	(11)
						F121A	+/–	–			(31)
						F121Y					L-870,810 (26)
V75P				(–)	(9)	S123A	++	++	+++	(+)	(1,9)
S81R;A	+/–	+/–	+/–	(–)	(5,6,9)	T125A*	+++	+++	+++	+ (+++)	(20,30)
P90D*	+++	–	–		(28)	T125K*					L-870,810 (26)
P90D/P145I	–	–	–		(28)	K127A				+	(3)
E92A;Q	+++	+++	+++	+++	(16)	A128T					S-1360 (27)
E92A;N	+	+	++		(17)	C130A*	+++	+++		++	OH-Coum (12,36)
E92K	+	+	+++	–	(16)						
T93A				(++)	(20)	C130G*				– (–)	OH-Coum (12,36)
G106A	+++	+++			(29)						
P109A;S*	+/–	+/–	–	+	(3,18,30)	C130S*				–	OH-Coum (13,14,36)
P109S/T125A	+++	+++	+++	(+/–)	(30)						
T112A	+++	+++			(18)	C130A/F185K/ C280S	+++	+++			OH-Coum (36)
H114E				++	(11)						

(*Continues*)

Table I (*Continued*)

Mutations	*3′-P*	*ST*	*Dis*	*Inf*	*Interaction (References)*[b]
C130S/F185K/C280S	+++	+			OH-Coum (36)
W131G/F185K/C280S	++	++			(36)
W132A;G;R//F185K/C280S	+++	−			OH-Coum (36)
W132Y/F185K/C280S	+++	++			OH-Coum (36)
I135P				(−)	(9)
K136A*	+/−	+/−	+++	−/++	(3,11,16)
K136E;R*	+++	+++	+++	+++	(16)
K136A/E138A	+++	+++	+++	+++	(16)
K136R//F185K/C280S	+++	++			(36)
E138A*				+	(3)
E138K*				++	S-1360 (11,27)
G140S*	+	+	+	Del	L-CA; L-731,988; (37)
G140S/F185K/C280S	+++	+++	+++		(38)
P142F				+++/(+++)	(39)
Y143F				Del	(3,32)
Y143N	+++		+++	Del	(3,32,33)
Y143G				+++/(+++)	(11,20,39)
N144K				−	(11)
N144Q				−/(−)	(39)
P145I*	−	−	−		(28)
P145A*				+	(11)
P145F*				−/(−)	(39)
P145I/F185K/C280S	−	−	−		(28)
Q146K				+	S-1360 (11,27)
S147I	−		+++	−	(32,33)
Q148A*	++	−			(15)
Q148K*				Del	(11)
Q148L*	+	++	+	Del	(11,17)
Q148N*	+++	++			(15)
Q148A/F185K/C280S	+	+			(36)
V150E	+++	+++	+++		(28)
V151A*				(+)	(9)
V151I*					L-870,810 (26)
V151D/E152Q	−	−	−	−	(10)
V151T/S153Q	++	−	+++		(40)
V151L/S153L	+++	−	−		(40)
V151L/E152V/S153F	++	−	−		(40)
V151A/E152M/S153A	++	−	−		(40)
V151Y/E152V/S153P	+++	−	−		(40)

V151H/E152S/ S153T	+++	–	–		(40)	N155L	+	+	–	Del	(11,17)
						N155S					L-870, 810 (26)
V151A/E152L/ S153T	+++	–	–		(40)	K156A*				+/+++/ (+++)	(3,39)
V151K/E152L/ S153T	++	–	–		(40)	K156E*	–	–	+/–	Del	(11,17,41)
V151S/E152S/S153P	++	–	–		(40)	K156R*					(42)
V151H/E152G/ S153L	+++	–	–		(40)	K156I*	–	–	–		(28)
						K156E/K159E	–	–	–	–	(11,41)
V151E/E152S/ S153N	++	–	–		(40)	K156A/E157A				+	(3)
						E157A/K159A				+	(3)
V151T/E152S/ S153M	+++	–	–		(40)	L158F	+++	+++	+++		(28)
						K159A*				++/(++)	(39)
V151T/E152F/ S153C	+++	–	–		(40)	K159E*	–	–	+++	–/Del	(11,41)
						K159N;S*	+	+	+		(17)
E152A;C;D;G;H;P; Q;V;K*	–	–	–	– (–)	(1,3,5,6,9, 11,17,18, 28,31, 33,34)	K159Q*	+++		+++		(33)
						K159R*					(42)
						K159A/K160A				+	(3)
						K159A;P;Q				(+)	(9,31,32)
E152A/K156A				–	(3)	K160A*				+++/ (+++)	(39)
E152N/S153R	++	–	–		(40)						
S153A;R*	+	+	++	++	(1,11)	K160D*					S-1360 (27)
S153A*	+++		+++	+++	S-1360 (27,32,33)	K160E*	+++	+++	+++	–	(41)
						V165A	++			– (–)	PIC (4,12)
S153Y*	++	++	++	+	L-708,906; L-731,988; L-CA (22,25,26)	V165I					S-1360 (27)
						R166A*				–	(3)
						R166T*	++	++			(42,43)
						R166A/D167A				–	(3)
M154I	+++	+++	+++	++	L-708,906; L-731,988; L-CA (22,25,26)	D167A*				+	(3)
						Q168A	+++	+++		(–)	LEDGF (44,45)
						Q168L	+++	–		(–)	LEDGF (44)
						Q168P	–	–		(–)	LEDGF (44)
N155E;K	–	–	–	Del	(11,17)						

(*Continues*)

Table I (*Continued*)

Mutations	*3′-P*	*ST*	*Dis*	*Inf*	*Interaction (References)*[b]
E170A/H171A	+++	+++		+/Del	(3,46)
E170A/K173A				+	(3)
H171A/K173A*				+	(3)
L172M*	+++	+++	+++		(28)
L172A/K173A	+++	+++		−	(46)
T174A	+	+		−	(46)
V176A/Q177A	+++	+++		Del	(46)
M178A	−	−		−	(46)
A179P				(−)	(9)
V180A/F181A	−	−		−	(46)
N184D;L				−	(11)
F185A;K;L;H*	+++	+++		(−)	(16)
K186A;Q;E	+++			− (−)	(12,20)
K187A				−	(12)
K188A				−	(12)
G189A	+++	+++			(18)
S195A				(++)	(20)
E198A/R199A				−	(3)
R199A;C*	+++	+++	+++	−/Del (−)	(3,5,6, 11,12)
R199E*				−	(11)
R199A/D202A				−	(3)
R199T/D202A				−	(3)
V201I					(27)
K211A*				(++)	(20)
K211A/E212A				+	(3)
Q214L/Q216L	+++			− (−)	(12)
K215A*				+++	(12)
K215A/K219A	++			− (−)	(12)
K219A*				+	(12)
N222A	++	++	++		(47)
F223A	++	++	++		mAb33 (47,48)
R224A	++	++	++		mAb33 (47,48)
Y226					mAb33 (48)
Y227A	++	++	++		(47)
R228A				− (−)	(49)
S230R*	+++	+++			L-708,906; S-1360 (23)
R231A	++	+	+	+	(47,49)
P233A	++	++	++		(47)
L;V234A	++	++	++	+++	(47,49)
W235A;E;F	+++	+++	+++	+/− (−)	PIC (2, 4–6,9)
K236A*				+++	(49)
K236E*				− (−)	(49)
K236A/K240A				−	(3)
K236/E246A				− (−)	(49)
L241A	−	−	−	− (−)	(47,49)
L242A	−	−	+	− (−)	(47,49)
W243A	++	++	++		(47)
K244A*				− (−)	(49)
K244E*	−	−		(−)	(50)
K244A/E246A				−	(3)
E246A*	++	++	++	Del (+)	(47,49)
E246K*				− (−)	(49)
D253A/D256A				+	(30
W243A	++	++	++		(47)

K244A*				– (–)	(49)	R263A*				Del	(49)
K244E*	–	–		(–)	(50)	R263L*	++	++	++		(47)
K244A/E246A				–	(3)	K264A*				++	(49)
E246A*	++	++	++	Del (+)	(47,49)	K264E*				– (–)	(49)
E246K*				– (–)	(49)	K266A;E				– (–)	(49)
D253A/D256A				+	(3)	I267					mAb33 (48)
N254A	++	++	++		(47)	I268					mAb33 (48)
K258A				– (–)	(49)	R269A*				Del (+)	(49)
V260E	–	–	–	– (–)	(47,49)	R269A/D270A				+	(3)
R262A*				++	(49)	K273A				+++	(49)
R262G*	++	++	+		(47)	C280S*	+++	+++			(14)
R262A/R263A				– (–)	(49)	S283A				(++)	(20)
R262A/K264A				– (–)	(49)	R284G;K	+++	+++			(51)
R262D/R263V/ K264E				– (–)	(49)						

[a]Abbreviations: 3′-P, 3′-processing; ST, strand transfer; Dis, disintegration; Inf, infectivity; PIC, pre-integration complex; Del, delayed; L-CA, L-chicoric acid; OH-Coum, hydroxycoumarin.

– = 0–10%, + = 10–40%, ++ = 40–80%, +++ =80–100%, *= mutant present elsewhere in the table as a combination / = separates differential results from independent publications.

[b]References: (1) Engelman and Craigie, 1992; (2) Engelman *et al*, 1995; (3) Wiskerchen, and Muesing, 1995; (4) Lu *et al*, 2005; (5) Leavitt, Shiue, and Varmus, 1993; (6) Leavitt *et al*, 1996; (7) Nakamura *et al*, 1997; (8) Nomura, Masuda, and Kawai, 2006; (9) Cannon *et al*, 1994; (10) LaFemina *et al*, 1992; (11) Lu *et al*, 2005; (12) Lu *et al*, 2004; (13) Zhu, Dobard, and Chow, 2004; (14) Bischerour *et al*, 2003; (15) Johnson *et al*, 2006; (16) Engelman et al, 1997; (17) Gerton et al, 1998; (18) Drelich, Wilhelm, and Mous, 1992; (19) Cherepanov *et al*, 2000; (20) Tsurutani *et al*, 2000; (21) Yoshinaga *et al*, 2002; (22) Hazuda *et al*, 2000; (23) Fikkert *et al*, 2003; (24) Svarovskaia *et al*, 2004; (25) Lee and Robinson, Jr., 2004; (26) Hazuda *et al*, 2004; (27) Fikkert *et al*, 2004; (28) Sayasith, Sauve, and Yelle, 2000; (29) Harper *et al*, 2001; (30) Taddeo *et al*, 1996; (31) Kulkosky *et al*, 1992; (32) Shin *et al*, 1994; (33) Oh *et al*, 1997; (34) Engelman, Bushman, and Craigie, 1993; (35) Parissi *et al*, 2000; (36) Al-Mawsawi *et al*, 2006; (37) King *et al*, 2003; King and Robinson, Jr., 1998; (38) Pluymers *et al*, 2000; (39) Ikeda *et al*, 2004; (40) Calmels *et al*, 2004; (41) Jenkins *et al*, 1997; (42) Drake *et al*, 1998; (43) Pilon *et al*, 2000; (44) Emiliani *et al*, 2005; (45) Vandekerckhove *et al*, 2006; (46) Priet *et al*, 2003; (47) Lutzke and Plasterk, 1998; (48) Ramcharan *et al*, 2006; (49) Lu, Ghory, and Engelman, 2005; (50) Williams *et al*, 2005; (51) Hickman, Dyda, and Craigie, 1997.

remains incomplete. In our opinion, the main function of the PIC is to separate the two reactions catalyzed by IN (3′-P and ST) into different cellular compartments over time *in vivo*, while *in vitro* these two reactions occur consequently without delay. It is plausible that IN may be kept inactive in the PIC until migration into the nucleus to prevent autointegration. A cellular cofactor present in the PIC, BAF, prevents autointegration (Zheng *et al.*, 2000). Other PIC-associated factors probably also keep IN inactive. For instance, HIV reverse transcriptase can inhibit IN catalytic activities *in vitro* (Oz *et al.*, 2002). The viral cDNA is protected from nucleases after isolation of PIC only with wild-type IN, whereas it is sensitive to nuclease digestion when the PIC is formed with IN mutant (Chen *et al.*, 1999; Miller *et al.*, 1997). Thus, IN is probably involved not only in 3′-P very early in the viral cycle but also in PIC formation. PIC formation could possibly be triggered by 3′-P completion. It is also likely that PIC rearrangements leading to the reactivation of IN, occur during the passage of the PIC through the nuclear envelope and/or its association with chromatin.

We will focus on two factors that are known to tether the viral cDNA to chromosomal host DNA, emerin, and LEDGF/p75. Recently, the interaction of viral cDNA with chromatin has been reported to be dependent on emerin, a nuclear protein associated with PIC through BAF. Both emerin and BAF are required for the appropriate localization of the viral cDNA in the nucleus before integration. However, emerin and BAF do not facilitate HIV integration (Jacque and Stevenson, 2006). Another molecular tether linking HIV-1 IN protein to chromatin is LEDGF/p75. Binding of LEDGF/p75 to IN targets IN to chromatin, and promotes ST. Failure of HIV replication in LEDGF/p75 knockdown cells suggests that LEDGF/p75 is a critical cofactor for efficient HIV integration. Disrupting its interactions with IN could be considered as a therapeutic strategy (Cherepanov *et al.*, 2003, 2005; Ciuffi *et al.*, 2005; Llano *et al.*, 2006; Maertens *et al.*, 2003; Vandekerckhove *et al.*, 2006).

IV. Approaches to Inhibit HIV Integration

A. Small Molecule Inhibitors of HIV IN Enzymatic Activities

Searching for enzymatic inhibitors of IN is straightforward. High throughput assays have been developed, and several *in vitro* assays are routinely used to elucidate the drug mechanisms of action (Marchand *et al.*, 2001). 3′-P assays monitor the release of the terminal dinucleotide from an oligonucleotide duplex mimicking the viral LTR ends whereas ST results in larger DNA molecules. Precleaved ("3′-processed") substrates are used to determine ST inhibition independently from 3′-P. Disintegration, the third IN-catalyzed reaction [the reverse of ST (Chow *et al.*, 1992)], can be

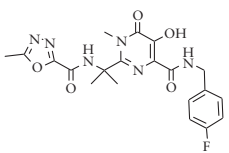
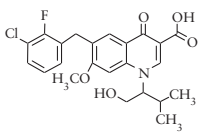
used to evaluate the site of drug action; as it is the only reaction that can be catalyzed by the IN catalytic core. Compounds that compete with target DNA within the enzyme catalytic site (Fig. 3b′) produce preferential inhibition of ST over 3′-P and are generally ineffective against disintegration (Espeseth *et al*., 2000). Those inhibitors are commonly referred to as "strand transfer inhibitors" (STI). In contrast, inhibitors that prevent the viral DNA binding to IN inhibit both 3′-P and ST with similar efficiency (Bonnenfant *et al*., 2004; Marchand *et al*., 2006b).

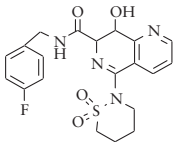
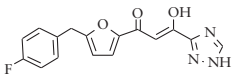
As it remains difficult to obtain drug–IN co-crystals, IN–DNA binding assays continue to be developed to investigate drug-binding sites in the IN–DNA complex. The Schiff base (Mazumder and Pommier, 1995) and disulfide cross-linking (Johnson *et al*., 2006) assays can be used to determine whether a given drug affects viral DNA binding to IN or alters crucial ST contacts between the IN-amino acid residue Q148 and the cytosine at the protruding viral DNA end (Johnson *et al*., 2007). A novel HIV IN inhibitor-binding site was discovered at the IN core dimer interface using photoaffinity labeling and mass spectrometric analysis (Al-Mawsawi *et al*., 2006).

The development of inhibitors has focused on targeting the D,D-35-E motif and chelating the divalent metal (Mg^{2+} vs Mn^{2+}) bound at the interface of the IN–DNA complex (Fig. 3; Semenova *et al*., 2006b). We have referred to this mode of inhibition as "interfacial inhibition" (Pommier and Cherfils, 2005; Pommier *et al*., 2005; Pommier and Marchand, 2005), as the drugs bind at the interface of two macromolecules (here IN and DNA) (Fig. 3b′) and trap a catalytic intermediate (here the 3′-P step), thereby preventing productive catalytic activity (here ST). Interfacial inhibition is commonly observed for a broad range of natural products targeting a variety of cellular targets (Pommier and Cherfils, 2005). Particular attention was given to the D,D-35-E motif after 5CITEP [a diketo acid (DKA)-like derivative] was first co-crystallized in the catalytic domain of HIV IN and shown to bind within the D,D-35-E motif (Goldgur *et al*., 1999). IN inhibitors currently in clinical trial (Table II) also contain DKA-like motifs that are believed to chelate divalent cations (Mg^{2+} or Mn^{2+}) within the D,D-35-E motif. Those drugs demonstrate preferential inhibition of the ST reaction. Preferential STI was first observed for caffeic acid phenethyl ester (CAPE) and proposed to be related to chelation of an IN divalent metal (Fesen *et al*., 1993). This model was further developed for the DKA derivatives, which were shown to act as competitors for the target (host chromosomal) DNA within the IN active site (Hazuda *et al*., 2000). The benefits of the STI emerged with the characterization of more potent DKA compounds effective against HIV infection (Tables II and III). IN residues involved in DKA and DKA-like resistance are listed in Table I.

As 3′-P is a prerequisite for ST and HIV integration, and is probably required for PIC formation, inhibiting 3′-P is a rational approach to inhibit

TABLE II Inhibitors of HIV-1 IN in Clinical Trials

Compound		*Preclinical studies*					
		Anti-IN activity IC_{50}, (μM)		*Antiviral activity*			
Name, source (references)[a]	*Structure*	*3′-P*	*ST*	EC_{50} (μM)	CC_{50} (μM)	*Comments*	*Clinical development status*
MK-0518, Raltegravir Insentress™ Merck & Co. (1,2,3,4)		[b]	0.016	0.017–0.029 (EC_{95})	[b]	Active against multidrug-resistance HIV-1 viruses. Oral bioavailability: rats (45%), dogs (69%), and rhesus monkey (8%). Human protein binding (82%). Does not inhibit the major cytochrome P450	In Phase III. Effective in heavily pretreated patients resistant to other treatments. Reduction of HIV counts: 1.7–2.2 log copies HIV-RNA/ml. Well tolerated
JTK-303, GS-9137, Elvitegravir Gilead Sciences, Inc. (5,6,7,8)		1–10	0.0072	9×10^{-4}	4.0	Active against drug-resistant clinical isolates of HIV-1 and HIV-2. Synergistic with 3TC and AZT/3TC; additive with AZT, Efavirenz, Indinavir, and Nelfinavir. Oral bioavailability: rats (34%) and dogs (30%)	In Phase I/II. Effective in heavily pretreated patients resistant to other treatments. Reduction of HIV counts: 1–2 log copies HIV-RNA/ml. Well tolerated

L-870,810, Merck & Co. (9,10)		0.25	0.015	0.015 to 0.1 (EC_{95})	>10	Active against multidrug-resistance HIV-1 viruses. Active against HIV-2 and SIV. Oral bioavailability: rats (41%), dogs (24%), rhesus (51%)	Stopped in Phase I/II due to toxicity in dogs. However well tolerated in patients. 1.7 log reduction of HIV-RNA copies/ml
S-1360/GW-10,781, Shionogi-GSK[c] (11,12,13)		0.02[d]		0.2	12	Active against a variety of clinical isolates and drug-resistant variants of HIV-1. Synergic with HIV reverse transcriptase and protease inhibitors	Stopped in Phase II for undisclosed scientific reasons. Well tolerated

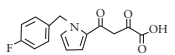
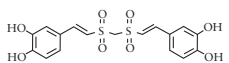
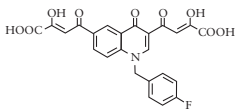
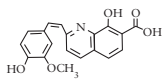
[a] References: (1) Morales-Ramirez *et al.* (2005); (2) Summa *et al.* (2006); (3) Laufer *et al.* (2006); (4) Markowitz *et al.* (2006); (5) Sato *et al.* (2006); (6) Matsuzaki *et al.* (2006); (7) Kawaguchi *et al.* (2006); (8) DeJesus *et al.* (2006); (9) Hazuda *et al.* (2004); (10) Little *et al.* (2005); (11) Fikkert *et al.* (2004); (12) Yoshinaga *et al.* (2002); (13) GlaxoSmithKline annual report (2003).

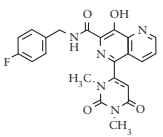
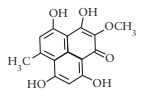
[b] Data unavailable from references.

[c] Joint venture Shionogi-GlaxoSmithKline Pharmaceuticals.

[d] No information regarding selectivity for 3′-P or ST.

TABLE III Representative Inhibitors of HIV-1 IN

		Anti-IN activity IC_{50} (μM)[a]		*Antiviral activity*		
Chemical family	*Structure*	*3′-P*	*ST*	*EC_{50} (μM)*	*CC_{50} (μM)*	*Comments (references)*[b]
Diketo acids (DKA)	 L-731,988	6	0.08	1	[c]	First representative of diketo acids. Archetype of a new ST selective inhibitors of HIV IN (1)
Chicoric acids	 Compound #4	4	5	2.4	187	Geminal disulfone analogue of chicoric acid. Time-of addition experiments indicated inhibition after reverse transcription (2)
Quinolin-4-one derivatives	 Cpd 8	0.44	0.016	4.29	>200	Newly designed bifunctional quinolonyl diketo acid derivative (3)
Styrylquinoline derivatives (SQL)	 FZ-41	2.8	3.7	1–4	300	Active against HIV-1 drug-resistant viruses. Inhibits migration of PIC into nucleus. Synergy with nevirapine, AZT (4,5)
Natural peptides (defensins)	ILPWKWPWWPWRR Indolicidin	60	57	(35–52 μM)	[c]	In spite of additional targets besides IN, direct binding to DNA represents a novel feature for IN inhibition (6,7,8)

Naphthyridine derivatives	 Compound #11	[c]	0.035	0.02–0.04 (EC_{95})	[c]	Good pharmacokinetics and oral bioavailability when dosed in rats and dogs (9)
Natural products	 Funalenone	[c]	10	1.7	87	Isolated from *Penicillium* sp. FKI-1463 (10)

[a] Abbreviations: IC_{50}, concentration required for 50% inhibition of HIV-1 integrase activity; EC_{50}, concentration required to induce the exponential growth of MT-2 cells infected by HIV by 50%; CC_{50}, cytotoxicity of compound.

[b] References: (1) Hazuda *et al.* (2000); (2) Meadows *et al.* (2005); (3) Di Santo *et al.* (2006); (4) Bonnenfant *et al.* (2004); (5) Mousnier *et al.* (2004); (6) Marchand *et al.* (2006b); (7) Robinson *et al.* (1998); (8); Krajewski *et al.* (2004); (9) Embrey *et al.* (2005); (10) Shiomi *et al.* (2005).

[c] Data not specified in references.

HIV replication. It might also be logical to combine 3′-P inhibitors with the currently developed ST inhibitors. A styrylquinoline (SQL) derivative, FZ-41, inhibits both 3′-P and ST with similar efficiency (Bonnenfant *et al.*, 2004), and has been confirmed as a cellular HIV IN inhibitor by developing drug-resistant viruses. The antiviral activity of FZ-41 could serve as a paradigm for 3′-P inhibitors that could also prevent PIC formation. Inhibition of the IN nuclear import after SQL treatment (Mousnier *et al.*, 2004) could be a consequence of PIC assembly failure.

B. Targeting the PIC

According to the paradigm of interfacial inhibition (Pommier and Cherfils, 2005; Pommier and Marchand, 2005), protein–protein interactions (IN monomer–IN monomer, IN–LEDGF/p75, IN–matrix, IN–INI1, matrix–BAF, etc.) and protein–DNA junctions (IN–viral DNA, BAF–viral DNA, etc.) within the PIC are equally important for integration. Alteration of any of these interfaces may prevent integration. For example, DKA-like inhibitors change the target DNA-binding surface within the IN-active site (protein–DNA interface) due to the chelation of divalent cations after 3′-P (Fig. 3b′). Another candidate target is LEDGF/p75, as HIV replication is markedly reduced in LEDGF/p75 knockdown cells due to absence of IN–LEDGF interaction (Vandekerckhove *et al.*, 2006). Therefore, prevention or alteration of macromolecular contacts among the PIC components is a rational and promising approach for the inhibition of HIV integration. Specific IN residues interacting with PIC components are highlighted in Table I. Assays developed to identify inhibitors of IN-enzymatic functions (protein–DNA contact) may not identify interfacial inhibitors because the interfacial contacts with PIC components are not required for IN enzymatic activities *in vitro* (Emiliani *et al.*, 2005). Such compounds could therefore be mistakenly ruled out during routine biochemical screening, although they still may alter *in vivo* PIC formation, which underlines the need to develop additional assays for integration inhibitors.

V. Inhibitors in Clinical Trials

The first pharmacological inhibitor in clinical trial was the guanosine quartet (AR-177, Zintevir, Aronex Pharmaceuticals, Inc.). In spite of it being identified initially as an IN inhibitor based on *in vitro* activity (Mazumder *et al.*, 1996a), this compound was also found to inhibit viral entry *in vivo*. AR-177 was discontinued after phase I/II clinical trial. The next two IN inhibitors in clinical trials (Savarino, 2006; Semenova *et al.*, 2006b) were a naphthyridine carboxamide derivative (L-870,810) (Merck & Co.) (Hazuda *et al.*, 2004; Little *et al.*, 2005) and a DKA derivative (S-1360/GW-810781)

(Shionogi–GlaxoSmithKline Pharmaceuticals) (GlaxoSmithKline, 2003; Yoshinaga *et al*., 2002; Table II). Both were discontinued after phase II trials. L-870,810 caused toxicity in dogs during long-term dosing. The reasons for termination of the clinical trial for S-1360 have not been fully disclosed (GlaxoSmithKline, 2003). However, their well-tolerated properties in humans (Table II) demonstrated the proof of concept for using of HIV-1 IN inhibitors as antiretrovirals.

Two IN inhibitors are currently in clinical trial: a derivative of quinolone antibiotics (JTK-303/GS-9137, Gilead Sciences, Inc.) (DeJesus *et al*., 2006; Kawaguchi *et al.,* 2006; Matsuzaki *et al*., 2006; Sato *et al*., 2006) and an STI from "Merck & Co." (MK-0518) (Laufer *et al*., 2006; Markowitz *et al*., 2006; Morales-Ramirez *et al*., 2005; Summa *et al*., 2006; Table II). Their efficacy and good tolerance in heavily pretreated patients that had failed reverse transcriptase and protease inhibitors (Table II) is encouraging, especially for patients living with multidrug-resistant HIV.

VI. Inhibitors in Preclinical Development

Reviews have dealt in detail with the development and progress in the design of IN inhibitors (Dayam *et al*., 2006; Johnson *et al*., 2004; Pommier *et al*., 2005; Savarino, 2006; Semenova *et al*., 2006b; Zhao *et al*., 2007). Therefore, no attempt is made here to list all the inhibitors. We will only summarize and review selected classes of HIV IN inhibitors as potential as drug leads (Table III). Table I also lists all the IN residues involved in drug-resistance mechanisms.

Screening of biologically active natural extracts (plant, microbial, fungi, marine organisms) continues to serve as a source for identifying new leads. A majority of reported IN inhibitors are derived from natural products. Examples include CAPE (Fesen *et al*., 1993), anthracyclines (Fesen *et al*., 1993), curcumins (Mazumder *et al*., 1995, 1997), flavones and flavonoids (Fesen *et al*., 1994; Rowley *et al*., 2002), lignans and lignaloids (Eich *et al*., 1996; Ovenden *et al*., 2004), depsides and depsidones (Neamati *et al*., 1997a), α-hydroxytropolones (Semenova *et al*., 2006a), lithospermic acid (Abd-Elazem *et al*., 2002), indolicidin (Krajewski *et al*., 2003, 2004; Marchand *et al*., 2006b), Chicoric acids (Meadows *et al*., 2005; Neamati *et al*., 1997b), integrasone (Herath *et al*., 2004), and coumarins (Mazumder *et al*., 1996a; Zhao *et al*., 1997). Despite the fact that many of these compounds inhibit other viral targets such as reverse transcriptase, protease, and gp120 (Mazumder *et al*., 1996b; Pluymers *et al*., 2000; Pommier and Neamati, 1999; Robinson *et al*., 1998; Semenova *et al*., 2006a), their structure–activity relationship demonstrated the importance of hydroxy groups for anti-IN activity as well as the suggestion of their possible mechanism of action as metal chelators (Fesen *et al*., 1994). An interesting approach consists of studying parallel structure–activity relationship with

closely related HIV targets such as IN and RNase H (Semenova *et al.*, 2006a). An identified new natural product IN inhibitor is funalenone (Shiomi *et al.*, 2005), isolated from *Penicillium* sp. FKI-1463 (Table III) shows good antiviral activity.

At least three strategies are currently used for the discovery of synthetic IN inhibitors: (1) chemical derivatives based on previously known IN inhibitors such as DKA (Barreca *et al.*, 2005; Di Santo *et al.*, 2005), naphthyridine (Embrey *et al.*, 2005; Guare *et al.*, 2006), SQL (Normand-Bayle *et al.*, 2005), L-CA (Charvat *et al.*, 2006), and α-hydroxytropolones (Budihas *et al.*, 2005; Didierjean *et al.*, 2005; Semenova *et al.*, 2006a); (2) three-dimensional pharmacophore searches based on previously discovered compounds (Deng *et al.*, 2006); (3) hybrid molecules comprising core structures of two or more known inhibitors [DKA–catechol (Maurin *et al.*, 2006), DKA–nucleobase scaffold hybrids (Nair *et al.*, 2006)]. At the same time, bifunctional compounds (that contain two identical active groups) provide a rationale for further work due to the potent inhibitory properties of a bifunctional DKA derivative (Cpd 8, Table III; Di Santo *et al.*, 2005) and of geminal disulfone analogues of the Chicoric acid (compound 4), Table III (Meadows *et al.*, 2005).

As IN functions as a multimer, dimerization inhibitors (Camarasa *et al.*, 2006) ought to be considered. However, the limitation is to develop assays that can unambiguously provide evidence for such a mechanism. Along the same lines, it is logical to consider drugs that bind at the interface of the macromolecular complexes formed by IN and cellular cofactors during integration. Inhibiting the IN–LEDGF interface would require the development of assays that monitor protein–protein interactions not only by reducing their formation but also by stabilizing/trapping abortive intermediates, as in the case of the interfacial inhibitors (Pommier and Cherfils, 2005; Pommier and Marchand, 2005).

VII. Perspectives

The major goal of anti-HIV therapy is the efficient suppression of viral load for as long as possible; that is, without emergence of resistant viruses. To achieve such a goal, it is rational to combine therapies targeting several viral targets. Virus-specific targets are always attractive because selective inhibitors should be devoid of side effects as the infected cells lack the viral-specific target.

After the initial discovery of IN in 1978 (Grandgenett *et al.*, 1978) and establishing its requirement for HIV replication (Hippenmeyer and Grandgenett, 1984), major discoveries have paved the way for the development of IN inhibitors. These include *in vitro* assays for integration (Bushman and Craigie, 1991; Craigie *et al.*, 1990, 1991; Fitzgerald *et al.*, 1991;

Katzman *et al*., 1989; Sherman and Fyfe, 1990); identification of IN domains and highly conserved residues (Engelman and Craigie, 1992; van Gent *et al*., 1993; Vink and Plasterk, 1993; Vink *et al*., 1993; Table I); determination of X-ray crystal structures of the core and C-domains (Bujacz *et al*., 1995; Chen *et al*., 2000; Dyda *et al*., 1994); elucidation of the solution of the structure of the N-domain (Cai *et al*., 1997); and the role of cellular cofactors in HIV integration (Cherepanov *et al*., 2005).

Promising results of clinical trials for IN inhibitors (DeJesus *et al*., 2006; Markowitz *et al*., 2006; Savarino, 2006) show the feasibility of using IN inhibitors as antiretroviral therapy. This tremendous achievement will prompt the development of new inhibitors based on the existing ones and on novel chemotypes. Obtaining co-crystal structures for the most effective and promising inhibitors is limited by the challenge of solving the structure of full-length IN bound to its DNA substrates (donor viral and acceptor target DNA duplexes). However, it is not excluded that the inhibitors themselves might help to elucidate such structures if they can act as interfacial inhibitor and trap stable macromolecular complexes. Together with the mapping of drug resistance IN mutations, these structures should provide rationale for further chemical modifications and improvement of the inhibitors. The search for clinically effective IN inhibitors include optimization of pharmacological parameters such as a reduced binding to human serum proteins and limited dependence on metabolitic activation pathways (Laufer *et al*., 2006). Finally, besides systemic therapies, topical IN inhibitors are worthwhile pursuing as curative and preventive therapies.

References

Abd-Elazem, I. S., Chen, H. S., Bates, R. B., and Huang, R. C. (2002). Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from Salvia miltiorrhiza. *Antiviral Res.* **55**, 91–106.

Al-Mawsawi, L. Q., Fikkert, V., Dayam, R., Witvrouw, M., Burke, T. R., Jr., Borchers, C. H., and Neamati, N. (2006). Discovery of a small-molecule HIV-1 integrase inhibitor-binding site. *Proc. Natl. Acad. Sci. USA* **103**, 10080–10085.

Barreca, M. L., Ferro, S., Rao, A., De Luca, L., Zappala, M., Monforte, A. M., Debyser, Z., Witvrouw, M., and Chimirri, A. (2005). Pharmacophore-based design of HIV-1 integrase strand-transfer inhibitors. *J. Med. Chem.* **48**, 7084–7088.

Bischerour, J., Leh, H., Deprez, E., Brochon, J. C., and Mouscadet, J. F. (2003). Disulfide-linked integrase oligomers involving C280 residues are formed *in vitro* and *in vivo* but are not essential for human immunodeficiency virus replication. *J. Virol.* **77**, 135–141.

Bonnenfant, S., Thomas, C. M., Vita, C., Subra, F., Deprez, E., Zouhiri, F., Desmaele, D., D'Angelo, J., Mouscadet, J. F., and Leh, H. (2004). Styrylquinolines, integrase inhibitors acting prior to integration: A new mechanism of action for anti-integrase agents. *J. Virol.* **78**, 5728–5736.

Budihas, S. R., Gorshkova, I., Gaidamakov, S., Wamiru, A., Bona, M. K., Parniak, M. A., Crouch, R. J., McMahon, J. B., Beutler, J. A., and Le Grice, S. F. (2005). Selective

inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones. *Nucleic Acids Res.* **33**, 1249–1256.

Bujacz, G., Jaskolski, M., Alexandratos, J., Wlodawer, A., Merkel, G., Katz, R. A., and Skalka, A. M. (1995). High-resolution structure of the catalytic domain of avian sarcoma virus integrase. *J. Mol. Biol.* **253**, 333–346.

Bushman, F. D., and Craigie, R. (1991). Activities of human immunodeficiency virus (HIV) integration protein in vitro: Specific cleavage and integration of HIV DNA. *Proc. Natl. Acad. Sci. USA* **88**, 1339–1343.

Cai, M., Zheng, R., Caffrey, M., Craigie, R., Clore, G. M., and Gronenborn, A. M. (1997). Solution structure of the N-terminal zinc binding domain of HIV-1 integrase. *Nat. Struct. Biol.* **4**, 567–577.

Calmels, C., de Soultrait, V. R., Caumont, A., Desjobert, C., Faure, A., Fournier, M., Tarrago-Litvak, L., and Parissi, V. (2004). Biochemical and random mutagenesis analysis of the region carrying the catalytic E152 amino acid of HIV-1 integrase. *Nucleic Acids Res.* **32**, 1527–1538.

Camarasa, M. J., Velazquez, S., San-Felix, A., Perez-Perez, M. J., and Gago, F. (2006). Dimerization inhibitors of HIV-1 reverse transcriptase, protease and integrase: A single mode of inhibition for the three HIV enzymes? *Antiviral Res.* **71**, 260–267.

Cannon, P. M., Wilson, W., Byles, E., Kingsman, S. M., and Kingsman, A. J. (1994). Human immunodeficiency virus type 1 integrase: Effect on viral replication of mutations at highly conserved residues. *J. Virol.* **68**, 4768–4775.

Charvat, T. T., Lee, D. J., Robinson, W. E., and Chamberlin, A. R. (2006). Design, synthesis, and biological evaluation of chicoric acid analogs as inhibitors of HIV-1 integrase. *Bioorg. Med. Chem.* **14**, 4552–4567.

Chen, H., Wei, S. Q., and Engelman, A. (1999). Multiple integrase functions are required to form the native structure of the human immunodeficiency virus type I intasome. *J. Biol. Chem.* **274**, 17358–17364.

Chen, J. C., Krucinski, J., Miercke, L. J., Finer-Moore, J. S., Tang, A. H., Leavitt, A. D., and Stroud, R. M. (2000). Crystal structure of the HIV-1 integrase catalytic core and C-terminal domains: A model for viral DNA binding. *Proc. Natl. Acad. Sci. USA* **97**, 8233–8238.

Cherepanov, P., Pluymers, W., Claeys, A., Proost, P., De Clercq, E., and Debyser, Z. (2000). High-level expression of active HIV-1 integrase from a synthetic gene in human cells. *Faseb. J.* **14**, 1389–1399.

Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., De Clercq, E., and Debyser, Z. (2003). HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *J. Biol. Chem.* **278**, 372–381.

Cherepanov, P., Ambrosio, A. L., Rahman, S., Ellenberger, T., and Engelman, A. (2005). Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. *Proc. Natl. Acad. Sci. USA* **102**, 17308–17313.

Chiu, T. K., and Davies, D. R. (2004). Structure and function of HIV-1 integrase. *Curr. Top. Med. Chem.* **4**, 965–977.

Chow, S. A., Vincent, K. A., Ellison, V., and Brown, P. O. (1992). Reversal of integration and DNA splicing mediated by integrase of human immunodeficiency virus. *Science* **255**, 723–726.

Ciuffi, A., Llano, M., Poeschla, E., Hoffmann, C., Leipzig, J., Shinn, P., Ecker, J. R., and Bushman, F. (2005). A role for LEDGF/p75 in targeting HIV DNA integration. *Nat. Med.* **11**, 1287–1289.

Craigie, R., Fujiwara, T., and Bushman, F. (1990). The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell* **62**, 829–837.

Craigie, R., Mizuuchi, K., Bushman, F. D., and Engelman, A. (1991). A rapid *in vitro* assay for HIV DNA integration. *Nucleic Acids Res.* **19**, 2729–2734.

Dayam, R., Deng, J., and Neamati, N. (2006). HIV-1 integrase inhibitors: 2003–2004 update. *Med. Res. Rev.* **26**, 271–309.

DeJesus, E., Berger, D., Markowitz, M., Cohen, C., Hawkins, T., Ruane, P., Elion, R., Farthing, C., Zhong, L., Cheng, A. K., McColl, D., and Kearney, B. P. (2006). Antiviral activity, pharmacokinetics, and dose response of the HIV-1 integrase inhibitor GS-9137 (JTK-303) in treatment-naive and treatment-experienced patients. *J. Acquir. Immune Defic. Syndr.* **43**, 1–5.

Deng, J., Kelley, J. A., Barchi, J. J., Sanchez, T., Dayam, R., Pommier, Y., and Neamati, N. (2006). Mining the NCI antiviral compounds for HIV-1 integrase inhibitors. *Bioorg. Med. Chem.* **14**, 3785–3792.

Didierjean, J., Isel, C., Querre, F., Mouscadet, J. F., Aubertin, A. M., Valnot, J. Y., Piettre, S. R., and Marquet, R. (2005). Inhibition of human immunodeficiency virus type 1 reverse transcriptase, RNase H, and integrase activities by hydroxytropolones. *Antimicrob. Agents Chemother.* **49**, 4884–4894.

Di Santo, R., Costi, R., Artico, M., Ragno, R., Greco, G., Novellino, E., Marchand, C., and Pommier, Y. (2005). Design, synthesis and biological evaluation of heteroaryl diketohexenoic and diketobutanoic acids as HIV-1 integrase inhibitors endowed with antiretroviral activity. *Farmaco* **60**, 409–417.

Di Santo, R., Costi, R., Roux, A., Artico, M., Lavecchia, A., Marinelli, L., Novellino, E., Palmisano, L., Andreotti, M., Amici, R., Galluzzo, C. M., Nencioni, L., *et al.* (2006). Novel bifunctional quinolonyl diketc acid derivations as HIV-1 integrase inhibitors: Design, synthesis, biological activities, and mechanism of action. *J. Med. Chem.* **49**, 1939–1945.

Drake, R. R., Neamati, N., Hong, H., Pilon, A. A., Sunthankar, P., Hume, S. D., Milne, G. W., and Pommier, Y. (1998). Identification of a nucleotide binding site in HIV-1 integrase. *Proc. Natl. Acad. Sci. USA* **95**, 4170–4175.

Drelich, M., Wilhelm, R., and Mous, J. (1992). Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein *in vitro*. *Virology* **188**, 459–468.

Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craigie, R., and Davies, D. R. (1994). Crystal structure of the catalytic domain of HIV-1 integrase: Similarity to other polynucleotidyl transferases. *Science* **266**, 1981–1986.

Eich, E., Pertz, H., Kaloga, M., Schulz, J., Fesen, M. R., Mazumder, A., and Pommier, Y. (1996). (-)-Arctigenin as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase. *J. Med. Chem.* **39**, 86–95.

Embrey, M. W., Wai, J. S., Funk, T. W., Homnick, C. F., Perlow, D. S., Young, S. D., Vacca, J. P., Hazuda, D. J., Felock, P. J., Stillmock, K. A., Witmer, M. V., Moyer, G., *et al.* (2005). A series of 5-(5,6)-dihydrouracil substituted 8-hydroxy-[1,6]naphthyridine-7-carboxylic acid 4-fluorobenzylamide inhibitors of HIV-1 integrase and viral replication in cells. *Bioorg. Med. Chem. Lett.* **15**, 4550–4554.

Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D., Vandekerckhove, L., Moisant, F., Ben-Slama, L., Witvrouw, M., Christ, F., Rain, J. C., *et al.* (2005). Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. *J. Biol. Chem.* **280**, 25517–25523.

Engelman, A., and Craigie, R. (1992). Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function *in vitro*. *J. Virol.* **66**, 6361–6369.

Engelman, A., Bushman, F. D., and Craigie, R. (1993). Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. *EMBO J.* **12**, 3269–3275.

Engelman, A., Englund, G., Orenstein, J. M., Martin, M. A., and Craigie, R. (1995). Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J. Virol.* **69**, 2729–2736.

Engelman, A., Liu, Y., Chen, H., Farzan, M., and Dyda, F. (1997). Structure-based mutagenesis of the catalytic domain of human immunodeficiency virus type 1 integrase. *J. Virol.* **71**, 3507–3514.

Espeseth, A. S., Felock, P., Wolfe, A., Witmer, M., Grobler, J., Anthony, N., Egbertson, M., Melamed, J. Y., Young, S., Hamill, T., Cole, J. L., and Hazuda, D. J. (2000). HIV-1 integrase inhibitors that compete with the target DNA substrate define a unique strand transfer conformation for integrase. *Proc. Natl. Acad. Sci. USA* **97**, 11244–11249.

Fesen, M. R., Kohn, K. W., Leteurtre, F., and Pommier, Y. (1993). Inhibitors of human immunodeficiency virus integrase. *Proc. Natl. Acad. Sci. USA* **90**, 2399–2403.

Fesen, M. R., Pommier, Y., Leteurtre, F., Hiroguchi, S., Yung, J., and Kohn, K. W. (1994). Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. *Biochem. Pharmacol.* **48**, 595–608.

Fikkert, V., Van Maele, B., Vercammen, J., Hantson, A., Van Remoortel, B., Michiels, M., Gurnari, C., Pannecouque, C., De Maeyer, M., Engelborghs, Y., De Clercq, E., Debyser, Z., *et al.* (2003). Development of resistance against diketo derivatives of human immunodeficiency virus type 1 by progressive accumulation of integrase mutations. *J. Virol.* **77**, 11459–11470.

Fikkert, V., Hombrouck, A., Van Remoortel, B., De Maeyer, M., Pannecouque, C., De Clercq, E., Debyser, Z., and Witvrouw, M. (2004). Multiple mutations in human immunodeficiency virus-1 integrase confer resistance to the clinical trial drug S-1360. *AIDS* **18**, 2019–2028.

Fitzgerald, M. L., Vora, A. C., and Grandgenett, D. P. (1991). Development of an acid-soluble assay for measuring retrovirus integrase 3′-OH terminal nuclease activity. *Anal. Biochem.* **196**, 19–23.

Gerton, J. L., Ohgi, S., Olsen, M., DeRisi, J., and Brown, P. O. (1998). Effects of mutations in residues near the active site of human immunodeficiency virus type 1 integrase on specific enzyme-substrate interactions. *J. Virol.* **72**, 5046–5055.

GlaxoSmithKline (2003). GlaxoSmithKline annual report. Brentford, Middlesex.

Goldgur, Y., Craigie, R., Cohen, G. H., Fujiwara, T., Yoshinaga, T., Fujishita, T., Sugimoto, H., Endo, T., Murai, H., and Davies, D. R. (1999). Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: A platform for antiviral drug design. *Proc. Natl. Acad. Sci. USA* **96**, 13040–13043.

Grandgenett, D. P., Vora, A. C., and Schiff, R. D. (1978). A 32,000-dalton nucleic acid-binding protein from avian retrovirus cores possesses DNA endonuclease activity. *Virology* **89**, 119–132.

Guare, J. P., Wai, J. S., Gomez, R. P., Anthony, N. J., Jolly, S. M., Cortes, A. R., Vacca, J. P., Felock, P. J., Stillmock, K. A., Schleif, W. A., Moyer, G., Gabryelski, L. J., *et al.* (2006). A series of 5-aminosubstituted 4-fluorobenzyl-8-hydroxy-[1,6]naphthyridine-7-carboxamide HIV-1 integrase inhibitors. *Bioorg. Med. Chem. Lett.* **16**, 2900–2904.

Harper, A. L., Skinner, L. M., Sudol, M., and Katzman, M. (2001). Use of patient-derived human immunodeficiency virus type 1 integrases to identify a protein residue that affects target site selection. *J. Virol.* **75**, 7756–7762.

Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* **287**, 646–650.

Hazuda, D. J., Anthony, N. J., Gomez, R. P., Jolly, S. M., Wai, J. S., Zhuang, L., Fisher, T. E., Embrey, M., Guare, J. P., Jr., Egbertson, M. S., Vacca, J. P., Huff, J. R., *et al.* (2004). A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proc. Natl. Acad. Sci. USA* **101**, 11233–11238.

Herath, K. B., Jayasuriya, H., Bills, G. F., Polishook, J. D., Dombrowski, A. W., Guan, Z., Felock, P. J., Hazuda, D. J., and Singh, S. B. (2004). Isolation, structure, absolute stereochemistry, and HIV-1 integrase inhibitory activity of integrasone, a novel fungal polyketide. *J. Nat. Prod.* **67**, 872–874.

Hickman, A. B., Dyda, F., and Craigie, R. (1997). Heterogeneity in recombinant HIV-1 integrase corrected by site-directed mutagenesis: The identification and elimination of a protease cleavage site. *Protein Eng.* **10**, 601–606.

Hippenmeyer, P. J., and Grandgenett, D. P. (1984). Requirement of the avian retrovirus pp32 DNA binding protein domain for replication. *Virology* **137**, 358–370.

Ikeda, T., Nishitsuji, H., Zhou, X., Nara, N., Ohashi, T., Kannagi, M., and Masuda, T. (2004). Evaluation of the functional involvement of human immunodeficiency virus type 1 integrase in nuclear import of viral cDNA during acute infection. *J. Virol.* **78**, 11563–11573.

Jacque, J. M., and Stevenson, M. (2006). The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. *Nature* **441**, 641–645.

Jenkins, T. M., Esposito, D., Engelman, A., and Craigie, R. (1997). Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. *EMBO J.* **16**, 6849–6859.

Johnson, A. A., Marchand, C., and Pommier, Y. (2004). HIV-1 integrase inhibitors: A decade of research and two drugs in clinical trial. *Curr. Top. Med. Chem.* **4**, 1059–1077.

Johnson, A. A., Marchand, C., Patil, S., Costi, R., Di Santo, R., Burke, T. R., and Pommier, Y. (2007). Probing HIV-1 Integrase Inhibitor Binding Sites with Position-Specific Integrase-DNA Crosslinking Assays. *Mol. Pharmacol.* **71**, 893–901

Johnson, A. A., Santos, W., Pais, G. C., Marchand, C., Amin, R., Burke, T. R., Jr., Verdine, G., and Pommier, Y. (2006). Integration requires a specific interaction of the donor DNA terminal 5′-cytosine with glutamine 148 of the HIV-1 integrase flexible loop. *J. Biol. Chem.* **281**, 461–467.

Katzman, M., Katz, R. A., Skalka, A. M., and Leis, J. (1989). The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the in vivo sites of integration. *J. Virol.* **63**, 5319–5327.

Kawaguchi, I., Ishikawa, T., Ishibashi, M., Irie, S., and Kakee, A. (2006). Safety and pharmacokinetics of single oral dose of JTK-303/GS-9137 a novel HIV integrase inhibitor. *In* "Healthy Volunteers 13th Conference on Retroviruses and Opportunistic Infections, Denver," February 5–9, 2006, poster no. 580, Foundation for Retrovirology and Human Health, Alexandria, VA.

King, P. J., and Robinson, W. E., Jr. (1998). Resistance to the anti-human immunodeficiency virus type 1 compound L-chicoric acid results from a single mutation at amino acid 140 of integrase. *J. Virol.* **72**, 8420–8424.

King, P. J., Lee, D. J., Reinke, R. A., Victoria, J. G., Beale, K., and Robinson, W. E., Jr. (2003). Human immunodeficiency virus type-1 integrase containing a glycine to serine mutation at position 140 is attenuated for catalysis and resistant to integrase inhibitors. *Virology* **306**, 147–161.

Krajewski, K., Long, Y. Q., Marchand, C., Pommier, Y., and Roller, P. P. (2003). Design and synthesis of dimeric HIV-1 integrase inhibitory peptides. *Bioorg. Med. Chem. Lett.* **13**, 3203–3205.

Krajewski, K., Marchand, C., Long, Y. Q., Pommier, Y., and Roller, P. P. (2004). Synthesis and HIV-1 integrase inhibitory activity of dimeric and tetrameric analogs of indolicidin. *Bioorg. Med. Chem. Lett.* **14**, 5595–5598.

Kulkosky, J., Jones, K. S., Katz, R. A., Mack, J. P., and Skalka, A. M. (1992). Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. *Mol. Cell. Biol.* **12**, 2331–2338.

LaFemina, R. L., Schneider, C. L., Robbins, H. L., Callahan, P. L., LeGrow, K., Roth, E., Schleif, W. A., and Emini, E. A. (1992). Requirement of active human immunodeficiency

virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. *J. Virol.* **66**, 7414–7419.

Laufer, R., Gonzalez-Paz, O. G., Taliani, M., Di Marco, A., Moteagudo, E., Bonelli, F., Fiore, F., Fonsi, M., Naimo, F., Verdirame, M., Alfieri, A., Marcucci, I., *et al.* (2006). Preclinical pharmacokinetics and metabolism of MK-0518, a potent HIV Integrase inhibitor in phase III clinical trial. *In* "16th AIDS International Conference", Toronto, Abstract THPE0027.

Leavitt, A. D., Shiue, L., and Varmus, H. E. (1993). Site-directed mutagenesis of HIV-1 integrase demonstrates differential effects on integrase functions *in vitro*. *J. Biol. Chem.* **268**, 2113–2119.

Leavitt, A. D., Robles, G., Alesandro, N., and Varmus, H. E. (1996). Human immunodeficiency virus type 1 integrase mutants retain *in vitro* integrase activity yet fail to integrate viral DNA efficiently during infection. *J. Virol.* **70**, 721–728.

Lee, D. J., and Robinson, W. E., Jr. (2004). Human immunodeficiency virus type 1 (HIV-1) integrase: Resistance to diketo acid integrase inhibitors impairs HIV-1 replication and integration and confers cross-resistance to L-chicoric acid. *J. Virol.* **78**, 5835–5847.

Little, S., Drusano, G., Schooley, R., Haas, D., Kumar, P., Hammer, S., McMahon, D., Squires, K., Asfour, R., Richman, D., Chen, J., Saah, A., *et al.* (2005). Antiretroviral effect of L-000870810, a novel HIV-1 integrase inhibitor, in HIV-1-infected patients. *In* "12th Conference on Retroviruses and Opportunistic Infections," February 22–25, 2005, Boston, MA, USA, Abstract 161.

Llano, M., Saenz, D. T., Meehan, A., Wongthida, P., Peretz, M., Walker, W. H., Teo, W., and Poeschla, E. M. (2006). An essential role for LEDGF/p75 in HIV integration. *Science* **314**, 461–464.

Lu, R., Limon, A., Devroe, E., Silver, P. A., Cherepanov, P., and Engelman, A. (2004). Class II integrase mutants with changes in putative nuclear localization signals are primarily blocked at a postnuclear entry step of human immunodeficiency virus type 1 replication. *J. Virol.* **78**, 12735–12746.

Lu, R., Ghory, H. Z., and Engelman, A. (2005a). Genetic analyses of conserved residues in the carboxyl-terminal domain of human immunodeficiency virus type 1 integrase. *J. Virol.* **79**, 10356–10368.

Lu, R., Limon, A., Ghory, H. Z., and Engelman, A. (2005b). Genetic analyses of DNA-binding mutants in the catalytic core domain of human immunodeficiency virus type 1 integrase. *J. Virol.* **79**, 2493–2505.

Lu, R., Vandegraaff, N., Cherepanov, P., and Engelman, A. (2005c). Lys-34, dispensable for integrase catalysis, is required for preintegration complex function and human immunodeficiency virus type 1 replication. *J. Virol.* **79**, 12584–12591.

Lutzke, R. A., and Plasterk, R. H. (1998). Structure-based mutational analysis of the C-terminal DNA-binding domain of human immunodeficiency virus type 1 integrase: Critical residues for protein oligomerization and DNA binding. *J. Virol.* **72**, 4841–4848.

Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J. Biol. Chem.* **278**, 33528–33539.

Marchand, C., Neamati, N., and Pommier, Y. (2001). *In vitro* human immunodeficiency virus type 1 integrase assays. *Meth. Enzymol.* **340**, 624–633.

Marchand, C., Johnson, A. A., Semenova, E., and Pommier, Y. (2006a). Mechanism and inhibition of HIV integration. *Drug. Discov. Today: Disease Mechanism* **3**, 253–260.

Marchand, C., Krajewski, K., Lee, H. F., Antony, S., Johnson, A. A., Amin, R., Roller, P., Kvaratskhelia, M., and Pommier, Y. (2006b). Covalent binding of the natural antimicrobial peptide indolicidin to DNA abasic sites. *Nucleic Acids Res.* **34**, 5157–5165.

Markowitz, M., Morales-Ramirez, J. O., Nguyen, B. Y., Kovacs, C. M., Steigbigel, R. T., Cooper, D. A., Liporace, R., Schwartz, R., Isaacs, R., Gilde, L. R., Wenning, L., Zhao, J., *et al.* (2006). Antiretroviral activity, pharmacokinetics, and tolerability of MK-0518, a

novel inhibitor of HIV-1 integrase, dosed as monotherapy for 10 days in treatment-naive HIV-1-infected individuals. *J. Acquir. Immune Defic. Syndr.* **43**, 509–515.

Matsuzaki, Y., Watanabe, W., Yamataka, K., Sato, M., Enya, S., Kano, M., Kodama, E., Matsuoka, M., and Ikeda, S. (2006). JTK-303/GS-9137, a novel small molecule inhibitor of HIV-1 integrase: Anti-HIV activity profile and pharmacokinetics in animals. *In* "13th Conference on Retroviruses and Opportunistic Infections, Denver," February 5–9, 2006, poster no 508, Foundation for Retrovirology and Human Health, Alexandria, VA.

Maurin, C., Bailly, F., Mbemba, G., Mouscadet, J. F., and Cotelle, P. (2006). Design, synthesis, and anti-integrase activity of catechol-DKA hybrids. *Bioorg. Med. Chem.* **14**, 2978–2984.

Mazumder, A., and Pommier, Y. (1995). Processing of deoxyuridine mismatches and abasic sites by human immunodeficiency virus type-1 integrase. *Nucleic Acids Res.* **23**, 2865–2871.

Mazumder, A., Gupta, M., Perrin, D. M., Sigman, D. S., Rabinovitz, M., and Pommier, Y. (1995). Inhibition of human immunodeficiency virus type 1 integrase by a hydrophobic cation: The phenanthroline-cuprous complex. *AIDS Res. Hum. Retroviruses* **11**, 115–125.

Mazumder, A., Neamati, N., Ojwang, J. O., Sunder, S., Rando, R. F., and Pommier, Y. (1996a). Inhibition of the human immunodeficiency virus type 1 integrase by guanosine quartet structures. *Biochemistry* **35**, 13762–13771.

Mazumder, A., Wang, S., Neamati, N., Nicklaus, M., Sunder, S., Chen, J., Milne, G. W., Rice, W. G., Burke, T. R., Jr., and Pommier, Y. (1996b). Antiretroviral agents as inhibitors of both human immunodeficiency virus type 1 integrase and protease. *J. Med. Chem.* **39**, 2472–2481.

Mazumder, A., Neamati, N., Sunder, S., Schulz, J., Pertz, H., Eich, E., and Pommier, Y. (1997). Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanisms of drug action. *J. Med. Chem.* **40**, 3057–3063.

Meadows, D. C., Mathews, T. B., North, T. W., Hadd, M. J., Kuo, C. L., Neamati, N., and Gervay-Hague, J. (2005). Synthesis and biological evaluation of geminal disulfones as HIV-1 integrase inhibitors. *J. Med. Chem.* **48**, 4526–4534.

Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997). Human immunodeficiency virus type 1 preintegration complexes: Studies of organization and composition. *J. Virol.* **71**, 5382–5390.

Morales-Ramirez, J. O., Teppler, H., Kovacs, C., Steigbigel, R. T., Cooper, D., Liporace, R. L., Schwartz, R., Wenning, L., Zhao, J., Gilde, L., Isaacs, R., and Nguyen, B. (2005). Antiretroviral effect of MK-0518, a novel HIV-1 integrase inhibitor, in ART-naïve HIV-infected patients. *In* "Program and Abstracts of the 10th European AIDS Conference, Dublin," November 17–20, 2005, European AIDS Clinical Society, Paris, pp. 83–92. Abstract LBPS1/6.

Mousnier, A., Leh, H., Mouscadet, J. F., and Dargemont, C. (2004). Nuclear import of HIV-1 integrase is inhibited *in vitro* by styrylquinoline derivatives. *Mol. Pharmacol.* **66**, 783–788.

Nair, V., Chi, G., Ptak, R., and Neamati, N. (2006). HIV integrase inhibitors with nucleobase scaffolds: Discovery of a highly potent anti-HIV agent. *J. Med. Chem.* **49**, 445–447.

Nakamura, T., Masuda, T., Goto, T., Sano, K., Nakai, M., and Harada, S. (1997). Lack of infectivity of HIV-1 integrase zinc finger-like domain mutant with morphologically normal maturation. *Biochem. Biophys. Res. Commun.* **239**, 715–722.

Neamati, N., Hong, H., Mazumder, A., Wang, S., Sunder, S., Nicklaus, M. C., Milne, G. W., Proksa, B., and Pommier, Y. (1997a). Depsides and depsidones as inhibitors of HIV-1 integrase: Discovery of novel inhibitors through 3D database searching. *J. Med. Chem.* **40**, 942–951.

Neamati, N., Hong, H., Sunder, S., Milne, G. W., and Pommier, Y. (1997b). Potent inhibitors of human immunodeficiency virus type 1 integrase: Identification of a novel

four-point pharmacophore and tetracyclines as novel inhibitors. *Mol. Pharmacol.* **52**, 1041–1055.

Nomura, Y., Masuda, T., and Kawai, G. (2006). Structural analysis of a mutant of the HIV-1 integrase zinc finger domain that forms a single conformation. *J. Biochem.* **139**, 753–759.

Normand-Bayle, M., Benard, C., Zouhiri, F., Mouscadet, J. F., Leh, H., Thomas, C. M., Mbemba, G., Desmaele, D., and d'Angelo, J. (2005). New HIV-1 replication inhibitors of the styryquinoline class bearing aroyl/acyl groups at the C-7 position: Synthesis and biological activity. *Bioorg. Med. Chem. Lett.* **15**, 4019–4022.

Oh, J. W., Oh, Y. T., Kim, D. J., and Shin, C. G. (1997). Characterization of human immunodeficiency virus type 1 integrase mutants expressed in *Escherichia coli*. *Mol. cells* **7**, 688–693.

Ovenden, S. P., Yu, J., Wan, S. S., Sberna, G., Tait, R. M., Rhodes, D., Cox, S., Coates, J., Walsh, N. G., and Meurer-Grimes, B. M. (2004). Globoidnan A: A lignan from Eucalyptus globoidea inhibits HIV integrase. *Phytochemistry* **65**, 3255–3259.

Oz, I., Avidan, O., and Hizi, A. (2002). Inhibition of the integrases of human immunodeficiency viruses type 1 and type 2 by reverse transcriptases. *Biochem. J.* **361**, 557–566.

Parissi, V., Caumont, A. B., de Soultrait, V. R., Calmels, C., Pichuantes, S., Litvak, S., and Dupont, C. H. (2000). Selection of amino acid substitutions restoring activity of HIV-1 integrase mutated in its catalytic site using the yeast *Saccharomyces cerevisiae*. *J. Mol. Biol.* **295**, 755–765.

Pilon, A. A., Marchand, C., Kavlick, M. F., Bajaj, K., Owen, J., Mitsuya, H., and Pommier, Y. (2000). Mutations in the HIV type 1 integrase of patients receiving long-term dideoxynucleoside therapy do not confer resistance to zidovudine. *AIDS Res. Hum. Retroviruses* **16**, 1417–1422.

Pluymers, W., Neamati, N., Pannecouque, C., Fikkert, V., Marchand, C., Burke, T. R., Jr., Pommier, Y., Schols, D., De Clercq, E., Debyser, Z., and Witvrouw, M. (2000). Viral entry as the primary target for the anti-HIV activity of chicoric acid and its tetra-acetyl esters. *Mol. Pharmacol.* **58**, 641–648.

Pommier, Y., and Cherfils, J. (2005). Interfacial inhibition of macromolecular interactions: Nature's paradigm for drug discovery. *Trends Pharmacol. Sci.* **26**, 138–145.

Pommier, Y., and Marchand, C. (2005). Interfacial inhibitors of protein-nucleic acid interactions. *Curr. Med. Chem. Anticancer Agents* **5**, 421–429.

Pommier, Y., and Neamati, N. (1999). Inhibitors of human immunodeficiency virus integrase. *Adv. Virus Res.* **52**, 427–458.

Pommier, Y., Johnson, A. A., and Marchand, C. (2005). Integrase inhibitors to treat HIV/AIDS. *Nat. Rev. Drug Discov.* **4**, 236–248.

Priet, S., Navarro, J. M., Querat, G., and Sire, J. (2003). Reversion of the lethal phenotype of an HIV-1 integrase mutant virus by overexpression of the same integrase mutant protein. *J. Biol. Chem.* **278**, 20724–20730.

Ramcharan, J., Colleluori, D. M., Merkel, G., Andrake, M. D., and Skalka, A. M. (2006). Mode of inhibition of HIV-1 Integrase by a C-terminal domain-specific monoclonal antibody. *Retrovirology* **3**, 34[electronic resource].

Robinson, W. E., Jr., McDougall, B., Tran, D., and Selsted, M. E. (1998). Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. *J. Leukoc. Biol.* **63**, 94–100.

Rowley, D. C., Hansen, M. S., Rhodes, D., Sotriffer, C. A., Ni, H., McCammon, J. A., Bushman, F. D., and Fenical, W. (2002). Thalassiolins A-C: New marine-derived inhibitors of HIV cDNA integrase. *Bioorg. Med. Chem.* **10**, 3619–3625.

Sato, M., Motomura, T., Aramaki, H., Matsuda, T., Yamashita, M., Ito, Y., Kawakami, H., Matsuzaki, Y., Watanabe, W., Yamataka, K., Ikeda, S., Kodama, E., *et al.* (2006). Novel HIV-1 integrase inhibitors derived from quinolone antibiotics. *J. Med. Chem.* **49**, 1506–1508.

Savarino, A. (2006). A historical sketch of the discovery and development of HIV-1 integrase inhibitors. *Expert. Opin. Investig. Drugs* **15**, 1507–1522.

Sayasith, K., Sauve, G., and Yelle, J. (2000). Characterization of mutant HIV-1 integrase carrying amino acid changes in the catalytic domain. *Mol. cells* **10**, 525–532.

Semenova, E. A., Johnson, A. A., Marchand, C., Davis, D. A., Yarchoan, R., and Pommier, Y. (2006a). Preferential inhibition of the magnesium-dependent strand transfer reaction of HIV-1 integrase by {alpha}-hydroxytropolones. *Mol. Pharmacol.* **69**, 1454–1460.

Semenova, E. A., Johnson, A. A., Marchand, C., and Pommier, Y. (2006b). Integration of human immunodeficiency virus as a target for antiviral therapy. *Curr. Opin. HIV AIDS* **1**, 380–387.

Sherman, P. A., and Fyfe, J. A. (1990). Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. *Proc. Natl. Acad. Sci. USA* **87**, 5119–5123.

Shin, C. G., Taddeo, B., Haseltine, W. A., and Farnet, C. M. (1994). Genetic analysis of the human immunodeficiency virus type 1 integrase protein. *J. Virol.* **68**, 1633–1642.

Shiomi, K., Matsui, R., Isozaki, M., Chiba, H., Sugai, T., Yamaguchi, Y., Masuma, R., Tomoda, H., Chiba, T., Yan, H., Kitamura, Y., Sugiura, W., *et al.* (2005). Fungal phenalenones inhibit HIV-1 integrase. *J. Antibiot. (Tokyo)* **58**, 65–68.

Sierra, S., Kupfer, B., and Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. *J. Clin. Virol.* **34**, 233–244.

Summa, V., Pace, P., Petroccci, A., Laufer, R., Cortese, R., Hazuda, D. J., Vacca, J. P., Young, S. D., Scheif, W. B., Miller, M. D., and Rowley, M. (2006). Discovery of MK-0518 a novel, potent and selective HIV integrase inhibitor in phase III clinical trials. *In* "16th AIDS International Conference," Toronto, Abstract THPE0020.

Svarovskaia, E. S., Barr, R., Zhang, X., Pais, G. C., Marchand, C., Pommier, Y., Burke, T. R., Jr., and Pathak, V. K. (2004). Azido-containing diketo acid derivatives inhibit human immunodeficiency virus type 1 integrase *in vivo* and influence the frequency of deletions at two-long-terminal-repeat-circle junctions. *J. Virol.* **78**, 3210–3222.

Taddeo, B., Carlini, F., Verani, P., and Engelman, A. (1996). Reversion of a human immunodeficiency virus type 1 integrase mutant at a second site restores enzyme function and virus infectivity. *J. Virol.* **70**, 8277–8284.

Tsurutani, N., Kubo, M., Maeda, Y., Ohashi, T., Yamamoto, N., Kannagi, M., and Masuda, T. (2000). Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells. *J. Virol.* **74**, 4795–4806.

Vandekerckhove, L., Christ, F., Van Maele, B., De Rijck, J., Gijsbers, R., Van den Haute, C., Witvrouw, M., and Debyser, Z. (2006). Transient and stable knockdown of the integrase cofactor LEDGF/p75 reveals its role in the replication cycle of human immunodeficiency virus. *J. Virol.* **80**, 1886–1896.

van Gent, D. C., Vink, C., Groeneger, A. A., and Plasterk, R. H. (Gent 1993). Complementation between HIV integrase proteins mutated in different domains. *EMBO J.* **12**, 3261–3267.

Van Maele, B., and Debyser, Z. (Maele 2005). HIV-1 integration: An interplay between HIV-1 integrase, cellular and viral proteins. *AIDS Rev.* **7**, 26–43.

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F., and Debyser, Z. (Maele 2006). Cellular co-factors of HIV-1 integration. *Trends Biochem. Sci.* **31**, 98–105.

Vink, C., and Plasterk, R. H. (1993). The human immunodeficiency virus integrase protein. *Trends Genet.* **9**, 433–438.

Vink, C., Groenink, M., Elgersma, Y., Fouchier, R. A., Tersmette, M., and Plasterk, R. H. (1990). Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. *J. Virol.* **64**, 5626–5627.

Vink, C., van Gent, D. C., Elgersma, Y., and Plasterk, R. H. (1991). Human immunodeficiency virus integrase protein requires a subterminal position of its viral DNA recognition sequence for efficient cleavage. *J. Virol.* **65**, 4636–4644.

Vink, C., Oude Groeneger, A. M., and Plasterk, R. H. (1993). Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type I integrase protein. *Nucleic Acids Res.* **21**, 1419–1425.

Williams, K. L., Zhang, Y., Shkriabai, N., Karki, R. G., Nicklaus, M. C., Kotrikadze, N., Hess, S., Le Grice, S. F., Craigie, R., Pathak, V. K., and Kvaratskhelia, M. (2005). Mass spectrometric analysis of the HIV-1 integrase-pyridoxal 5′-phosphate complex reveals a new binding site for a nucleotide inhibitor. *J. Biol. Chem.* **280**, 7949–7955.

Wiskerchen, M., and Muesing, M. A. (1995). Human immunodeficiency virus type 1 integrase: Effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J. Virol.* **69**, 376–386.

Yoshinaga, T., Sato, A., Fujishita, T., and Fujiwara, T. (2002). S-1360: In vitro activity of a new HIV-1 integrase inhibitor in clinical development. *In* "Conf Retroviruses Opportunistic Infect," February 24–28, 2002, 9, Abstr. no. 8.

Zhao, G., Wang, C., Liu, C., and Lou, H. (2007). New Developments in Diketo-Containing inhibitors of HIV-1 integrase. *Mini Rev. Med. Chem.* **7**, 707–725.

Zhao, H., Neamati, N., Hong, H., Mazumder, A., Wang, S., Sunder, S., Milne, G. W., Pommier, Y., and Burke, T. R., Jr. (1997). Coumarin-based inhibitors of HIV integrase. *J. Med. Chem.* **40**, 242–249.

Zheng, R., Ghirlando, R., Lee, M. S., Mizuuchi, K., Krause, M., and Craigie, R. (2000). Barrier-to-autointegration factor (BAF) bridges DNA in a discrete, higher-order nucleoprotein complex. *Proc. Natl. Acad. Sci. USA* **97**, 8997–9002.

Zhu, K., Dobard, C., and Chow, S. A. (2004). Requirement for integrase during reverse transcription of human immunodeficiency virus type 1 and the effect of cysteine mutations of integrase on its interactions with reverse transcriptase. *J. Virol.* **78**, 5045–5055.

Jim A. Turpin*, Marco L. Schito†, Lisa M. Miller Jenkins‡, John K. Inman§, and Ettore Appella‡

*Preventions Sciences Program, Division of AIDS, NIAID, National Institutes of Health, Bethesda, Maryland 20892

†Henry M. Jackson Foundation for the Advancement of Military Medicine, Division of AIDS, NIAID, National Institutes of Health, Bethesda, Maryland 20892

‡Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

§Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Topical Microbicides: A Promising Approach for Controlling the AIDS Pandemic via Retroviral Zinc Finger Inhibitors

I. Chapter Overview

As the HIV/AIDS pandemic has become feminized over the last 25 years, with women and girls representing over 50% of the new infections, topical microbicides have been proposed as a method to prevent HIV transmission. Topical microbicides may consist of gels, creams, films, and other alternative solid dosage forms or devices that deliver inhibitors of HIV entry and

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56008-4

replication to vaginal, rectal, and/or penile mucosal surfaces. Although we are yet to realize the promise of a topical microbicide-based prevention strategy, the eminence of results from ongoing Phase III clinical trials has spurred efforts to ensure a vibrant microbicide development pipeline, generating new candidates to improve upon or compliment the first generation microbicides. We have developed a new class of topical microbicides targeting the mutationally intolerant HIV-1 nucleocapsid protein (NCp7) zinc fingers, leading to loss of HIV replication capacity and production of noninfectious virus. We present the rationale for development of neutral thioester-based, *S*-acyl-2-mercaptobenzamide, HIV-1 NCp7 zinc finger inhibitors as topical microbicide candidates and advance the hypothesis that their development could add substantially to the microbicide pipeline. We have identified this new class of microbicides as pluripotent (for multiple antiviral targets) HIV-specific virucides.

II. The AIDS Pandemic and the Rationale for a Microbicide

The year 2006 marked the 25th anniversary of the first reported case of the disease and pandemic the world has come to know as HIV/AIDS (Gayle, 2006). Joint United Nations Programme on HIV/AIDS (UNAIDS)/World Health Organization (WHO) now estimates that HIV has infected between 34 and 47 million individuals worldwide with an estimated 11,000 new infections daily. New infections can be split almost equally between men and women, with over 50% of the new infections in women occurring in young girls and women under the age of 25 years (UNAIDS, 2006). As the pandemic has progressed, it has become apparent that young girls and women are more susceptible to HIV infection than men. This circumstance has led to the realization that in some parts of the world the greatest risk factor for women to acquire AIDS is being heterosexual and married. Increased susceptibility of women and girls to HIV infection arises from increased biological susceptibility and, also, from a number of other factors (Chan, 2005). Among the latter are poverty and social standing of young girls and women within the given population/region/community. These factors contribute to the overall risk by increasing exposure through socially acceptable, unacceptable, and/or survival-related high-risk practices. Thus, effective control methods to halt the spread of HIV/AIDS need to embody prevention strategies that break the cycle of new infections both biologically and culturally. It has been proposed that one approach would be to put control of the prevention strategy into the hands of the vulnerable population in a manner that is culturally acceptable to them. Topical microbicides have been proposed as a prevention strategy that could achieve this goal (Stein, 1990).

Topical microbicides consist of single or multiple molecular entities that target cellular or viral components, preventing replication or inactivating

the virus, leading to decreased transmission and acquisition of HIV. Microbicide strategies may or may not include activity against sexually transmitted infections (STIs) that promote the acquisition or course of HIV infection. The active entities of a topical microbicide preparation may be delivered to vaginal (and/or rectal) mucosal surfaces in the form of gels, creams, and solid dosage formulations, or devices, like a vaginal ring, such that the dispersal of the active ingredient provides a barrier to HIV transmission. There are still many deficiencies in our understanding of the mechanism(s) of transmission and acquisition of HIV via the vaginal or rectal mucosa. We know that the infectious inocula, supplied via the ejaculate, contain both cell-free and cell-associated virus (Coombs *et al.*, 1998; Gupta *et al.*, 1997; Quayle *et al.*, 1997). Numerous studies have shown that infectious virus can be recovered from seminal fluid mononuclear cells, but endogenous antiviral factors in semen make determinations and recovery of infectious cell-free virus highly variable. Results from studies based on nonhuman primate models argue strongly for cell-free virus as the source of infection, and it has been shown that viral load correlates with transmission (Cohen and Pilcher, 2005; Pilcher *et al.*, 2004). Once deposited in the vagina, the virus or infected cell(s) must penetrate the epithelium of the reproductive tissues to reach the susceptible monocytic, dendritic, and T cells in the submucosa. In the case of the vagina and ectocervix, the squamous epithelium is keratinized and can be up to 50-cell-layers thick. In the endocervix, the epithelia transitions to a single layer of columnar cells. Additional defenses may include the barrier properties of cervical mucous, antiviral factors secreted by the innate immune system, and protective factors from commensal microflora (Cole, 2006; Miller and Shattock, 2003). The mechanism by which HIV circumvents these defenses is unknown, but microtrauma resulting from either intercourse and/or access to the submucosa via STI lesions has been identified as a potential route of entry. A number of studies have suggested that once access to susceptible cells in the submucosa is obtained, infection potentially occurs in a two-stage process with local infection of these susceptible cells in the tissue followed by rapid dissemination from the genital tract-associated mucosa to regional lymph nodes (Haase, 2005; Miller *et al.*, 2005; Pope and Haase, 2003; Zhang *et al.*, 1999). The identity of the first type of cell that becomes infected is still unknown. Some studies identify resting T cells as the first cells to be infected. Others show strong evidence for capture of virus by dendritic and Langerhans cells and/or infection of dendritic cells, either of which would facilitate infection of resting T cells through cell-to-cell interactions (Frank and Pope, 2002). Irrespective of the manner of transmission, a microbicide would need to protect against viral infection, possibly at multiple stages of infection.

Above all, a microbicide must be efficacious, safe (not promote the acquisition of HIV or other STIs), and be acceptable to the user. Additionally, to be compatible for global use, it should be inexpensive to produce and easy

to distribute and use. The microbicide field and desirable microbicide properties have been reviewed (Klasse *et al.*, 2006; Lederman *et al.*, 2006; Stone, 2002). Table I provides a general summary of some desirable and undesirable characteristics of microbicides. Currently, there are no clinically proven safe and effective topical microbicides available; several Phase II/IIB and III clinical trials are ongoing, and results should start to become available in late 2007 (Dhawan and Mayer, 2006; Stone, 2004; Weber *et al.*, 2005). Since the impact of a topical microbicide is yet to be determined, mathematical modeling has been used to assess the potential impact of a microbicide that has only 60% efficacy, which is believed to be reasonably achievable. This modeling was done with potentially real world conditions based on our knowledge of condom use (<20%) and the fact that ~20% of the sexual encounters occur under at-risk conditions. This model suggests that a topical microbicide could reduce the number of new HIV infections by ~2.5 million in 3 years (Foss *et al.*, 2003; Karmon *et al.*, 2003). Additional studies have demonstrated population-specific roles for microbicides, as well as the effect of STIs on the potential use of microbicides (Smith *et al.*, 2005; Vickerman *et al.*, 2006). Thus, identification of a safe, effective, and acceptable microbicide could have a significant impact on the HIV pandemic.

The holy grail of HIV prevention is an effective HIV vaccine strategy. It is accepted that an effective prophylactic vaccine could reduce new infections to those levels seen with other effective modern vaccines, that is, a few cases in thousands. We are yet to identify an effective or even partially effective vaccine candidate, let alone advance it through clinical trials to licensure, develop the worldwide network needed to manufacture a potentially complex delivery vector, distribute it, and finally vaccinate enough people to make a significant impact on the pandemic. Even the best estimates suggest that it could be a decade or more before this can be accomplished. In the meantime, we are faced with the burden of a large population of uninfected individuals in relationships with partners with known or unknown HIV status. Thus, even in the context of a vaccine, other prevention strategies will be critical during the time required for the reservoir of infection to be cleared, and may continue to be an option for individuals who, for economic or social reasons, will not or cannot be vaccinated. Topical microbicides represent not only an alternative to a vaccination strategy, but also can be part of a combined strategy that includes education, community involvement, and condom use to reduce HIV infection on a pandemic level.

III. Topical Microbicides in Preclinical Development

Currently there are no clinically proven, safe, and effective topical microbicides to prevent the transmission of HIV. However, preclinical development efforts have identified several candidates which have been

TABLE I Characteristics of an Ideal Microbicide

Property	*Acceptable*	*Unacceptable*
Safety	One to six or more times daily Long periods Reproductive safety	Cause epithelial disruption Induce inflammation Absorbed systemically
Effect	Fast Long duration Irreversible	Unstable Interval between application and coitus short (before or after)
Acceptability	Formulation stability Acceptable to both sex partners Unobtrusive/pleasurable	Messy, leaky Burning, itching Cumbersome Odor/taste?/color? Applicator trauma/discomfort
Availability	Contraceptive/noncontraceptive formulations Low cost Unlimited access Ease of scale-up and GMP production	Costly for use and/or manufacturing Regulated distribution (MD or health official) Requires special storage/transport
Uses	Vaginal and/or rectal Unlimited use Compatible with condoms and other STD preventatives	Complicated use
Activity	Effect STDs in ejaculate and cervicovaginal secretions Broadly active: HIV + other STDs	Effect vaginal (rectal) microbial ecology Enhance growth of STDs or secondary pathogens

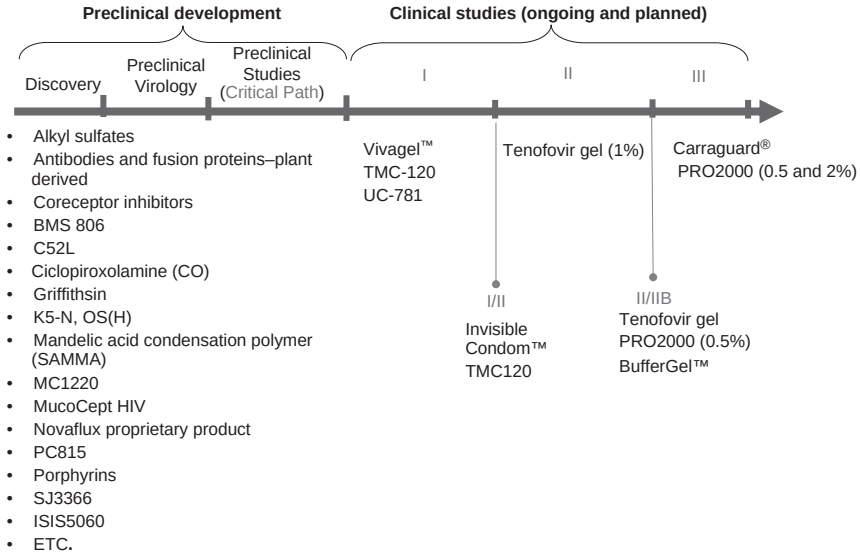


FIGURE 1 Microbicide candidates in development. Adapted from summaries provided by the Alliance for Microbicide Development (www.microbicide.org).

advanced and are currently undergoing clinical efficacy testing (Fig. 1). The candidates in both preclinical and clinical development have been extensively reviewed, so a candidate-by-candidate analysis will not be presented here (D'Cruz and Uckun, 2004; Klasse *et al.*, 2006; Lederman *et al.*, 2006; Stone, 2002; Turpin, 2003). The microbicide pipeline is routinely monitored and reported on by the Alliance for Microbicide Development in their Microbicide Resource Directory and other publications on their Web site (www.microbicide.org). Below, some key points about preclinical development of microbicides will be addressed and presented in the context of understanding the utility of HIV nucleocapsid protein (NCp7) inhibitors as potential microbicide candidates, which will be discussed later in the chapter.

There has been significant argument over the candidates selected for clinical advancement and the rationale for doing so. Five topical microbicide candidates, representing first generation strategies, were initially advanced to clinical efficacy assessments for the prevention of HIV transmission (Dhawan and Mayer, 2006; Stone, 2002). Recently, the surfactant-based microbicide Savvy® (C31G) Phase III clinical trials were stopped by their Data Safety Monitoring Boards (DSMB) when lower-than-expected incidence of HIV infection in their testing cohort was identified. No safety concerns were noted for Savvy®, leading to the conclusion that continuation of the trial was futile because of the increased numbers of participants

needed to provide a statistically relevant assessment of potential efficacy. The remaining clinical studies (PRO 2000, CarraguardTM, and Buffer-Gel^{TM}) have continued to accrue participants and are on track for completion. It is expected that unblinded analysis of these microbicide trials will begin in late 2007 at the earliest. Initial observations from these trials suggested that future trials/generations of microbicide candidates will need to adjust for potential changing HIV incidence, participant pregnancy rates, and subject adherence. Thus, although progress is being made toward the identification of a potential microbicide, we are still 5–7 years away from obtaining the information needed to focus preclinical development of microbicide strategies.

Preclinical microbicide development has advanced significantly in the last few years, leading to the identification of a number of microbicide candidates as well as the exploration of several potential targets for microbicide intervention (Table II). However, without a proof-of-concept for microbicide safety and efficacy, a great deal of this development is based on hypothetical "optimal" microbicide characteristics. The only evidence-based guidance for potential changing needs for the preclinical development of microbicides and microbicide targets has been the effect of the Col-1492 trial on preclinical microbicide development. Col-1492 used a microbicide gel containing the virucidal detergent Nonoxynol-9 that was found to increase the frequency of HIV transmission in a select population of high-frequency users (Centers for Disease Control and Prevention, 2000). This result caused two very significant changes in the field of preclinical microbicide science to occur. The first was a more intense focus on the safety of lead microbicides. A number of issues relevant to microbicide safety have been identified and addressed in recent reviews (D'Cruz and Uckun, 2004; Lard-Whiteford *et al.*, 2004; Turpin, 2003). These issues resulted in an

TABLE II Possible Targets for Microbicide Intervention

Target	*Subtarget*	*Candidates*
Virus	Nonspecific virucidals	Detergents, metal ions
	Specific virucidals	NCp7 inhibitors, Autocatalytic antibodies
Virus entry	Virus–cell interaction	Selectins, integrins oligosaccharides
	gp120-CD4	Antibodies, BMS806
	Gp41	C52L, T1249
	Coreceptor	CMPD167, TAK779
Reverse transcriptase	NNRTI	TMC120, UC781
	NRTI*	Tenofovir
Integrase	Speculative	

* NRTI, nucleoside reverse transcriptase inhibitor.

expansion of the types of *in vitro* (cell lines and primary cells), *ex vivo* (explants cervico-vaginal and rectal), and *in vivo* (murine and nonhuman primate) toxicity and efficacy models used to justify advancement to Phase I clinical testing (Catalone *et al.*, 2004; Cone *et al.*, 2006; Doncel *et al.*, 2004; Patton *et al.*, 2004).

The second effect of the Col-1492 trial on the microbicide field was the virtual elimination of virucidal agents from the microbicide pipeline which mediate their activity via nonspecific action on virus or cell membranes through a surfactant and detergent effect. The elimination of nonspecific virucides from consideration for development could be viewed as a positive outcome, that is, the removal of a class of candidates with potential safety issues. Consequently, however, there has been a general lack of focused development of specific, virucidal microbicide candidates that are less toxic. There are currently two lead virucidal microbicide strategies being pursued. The first is represented by the acid-buffering gels such as Amphora™ (Acidform) (Garg *et al.*, 2001) and BufferGel (Van de Wijgert *et al.*, 2001). This virucidal strategy takes advantage of the nonspecific effect of low pH (<4.5) on the infectivity of HIV-1 (loss of infectivity and inactivation) and other STIs by incorporating a buffering system in the microbicide gel to establish and maintain a lower vaginal pH (O'Connor *et al.*, 1995; Ongradi *et al.*, 1990). Although inherently a nonspecific virucidal approach, this strategy has potentially fewer safety concerns than a nonspecific detergent or surfactant-based virucide. The second virucidal strategy takes advantage of the physiochemical properties of microbicide candidates to identify target-specific interactions, leading to loss of infectivity by cell-free virus (virucidal activity). Examples of this type of virucide are cellulose acetate 1,2-benzenedicarboxylate [cellulose acetate phthalate (CAP)] (Boadi *et al.*, 2005; Lu *et al.*, 2006) and the nonnucleoside reverse transcriptase inhibitor (NNRTI) UC-781. The virucidal activity of CAP stems from its interaction with gp41, which leads ultimately to a nonfunctional six-helical bundle that prevents virus entry (Neurath *et al.*, 2001, 2002). In contrast, the virucide-like effects of UC-781 are mediated by its ability to penetrate cell-free virions, coupled with its high "on-rate" and low "off-rate" for the HIV-1 reverse transcriptase enzyme. Virucidal activity is the result of reduced reverse transcription capability, leading to inhibition of virus replication (Motakis and Parniak, 2002). Thus, although "microbicide virucides" are being identified following expansion of functional profiles of individual candidates or through broadly altering the vaginal microenvironment, focused efforts on the identification and development of less toxic candidates with anti-HIV virucidal activity as their primary mode of operation has lagged behind candidates targeting entry targets, coreceptors, and reverse transcriptase.

The Bill and Melinda Gates Foundation sponsored the Microbicide Development Strategy (MDS) in order to assure that microbicide development

has a strong foundation from which to address the need for new microbicides, as well as prepare for the outcomes of the current efficacy clinical trials (MDS Working Groups, 2006). The MDS brought together microbicide developers and interested parties to begin the process of identifying critical knowledge and infrastructure gaps in the field of microbicides. The MDS working groups focused on identifying the gaps that would prevent a sustained microbicide effort, given demonstrations of partial efficacy by one of the clinical candidates. A portion of these efforts specifically addressed preclinical development of microbicides and led to recognition that a priority gap in the microbicide field is "pipeline enhancement through rational development and acquisition of [new] chemical entities and targets" (Priority Gap 05). Thus, the MDS identified a continued need to identify additional microbicide targets as well as new chemical entities that have properties compatible with their use as microbicides.

IV. Characteristics of an Ideal Microbicide

Although we do not yet have a comprehensive picture of HIV transmission and infection, it is apparent from the brief introduction above that an optimal microbicide strategy would be one that could attack the virus at multiple stages of its life cycle or inactivate it. Classically, a multitarget approach to inhibition of HIV transmission and infection involves the development of a combination microbicide strategy. In this approach, microbicides are identified with complementary targets that could result in increased efficacy when administered simultaneously. Veazey *et al.* (2005) have shown that this approach is viable and can result in an additive or possible synergistic effect between individual microbicides to prevent transmission in a nonhuman primate model of simian/human immunodeficiency virus (SHIV) vaginal transmission. However, an alternative strategy would be to identify a single, pluripotent microbicide candidate that would be able to interdict HIV transmission and infection at multiple stages of viral replication via its antiviral target. Pluripotent microbicide activity is often approached by development of nonspecific compounds or strategies which act in a quick manner (<30 sec) to disrupt viral infectivity and cell function through disruption of viral and cell membranes. These approaches often result in unfavorable safety properties such as induction of proinflammatory cytokine production in exposed cells (Dezzutti *et al.*, 2004; Doncel *et al.*, 2004) and increased herpes simplex virus (HSV) susceptibility (Cone *et al.*, 2006). We have identified potentially pluripotent microbicide-compatible inhibitors of the HIV-1 NCp7 retroviral zinc fingers. Figure 2 summarizes the potential targets for microbicides during the HIV replication cycle overlaid with the stages at which NCp7 functions. This figure suggests that

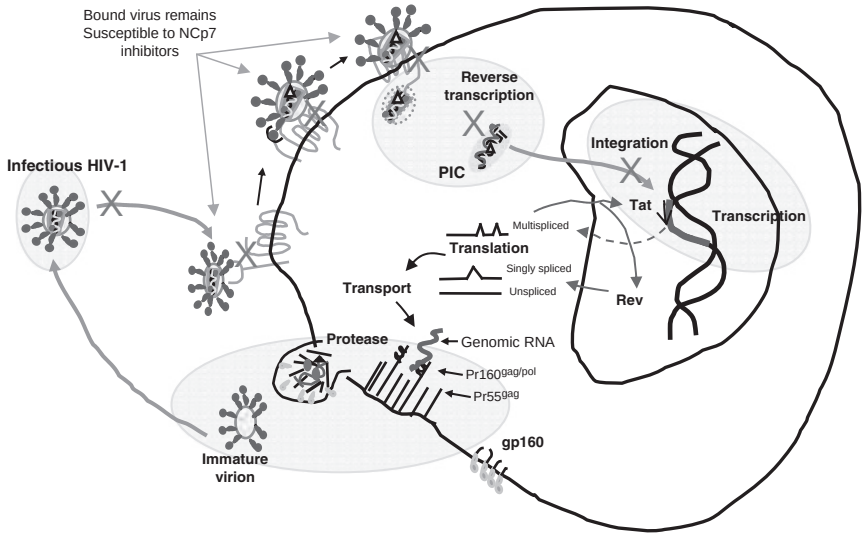


FIGURE 2 HIV-1 replication cycle. Xs indicate potential targets for topical microbicide intervention. Grayed areas identify segments in the replication cycle indicating stages where the HIV NCp7 or its zinc fingers play critical roles in HIV replication. These areas represent potential targets for an NCp7 zinc finger inhibitor-based strategy.

an NCp7 inhibitor of the appropriate characteristics could be a member of a new class of microbicide—a virucidal, pluripotent inhibitor of HIV replication.

V. The Retroviral Zinc Fingers of HIV-1 NCp7 as a Potential Microbicide Target

As described above, development of a specific virucidal compound with the ability to interfere with multiple stages of HIV replication has the potential to add significantly to the microbicide development pipeline. Although there are a number of conserved sequences and molecular targets in the HIV genome and its proteins (Pereira and Paridaen, 2004), the highly conserved and broadly functional retroviral zinc fingers of the HIV-1 NCp7 protein suggested that targeting this protein could represent a potentially potent attack point in virus replication.

NCp7 contains two highly conserved zinc fingers (Berg, 1986; Covey, 1986) derived from a Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys (CCHC) motif (Fig. 3A). These two retroviral zinc fingers make up the main structural elements of the NCp7 proteins, with each finger forming a tight NH–S turn connected by a short, flexible linker while the termini remain disordered (Lee *et al.*, 1998; South *et al.*, 1991). Within this motif, the Cys and His

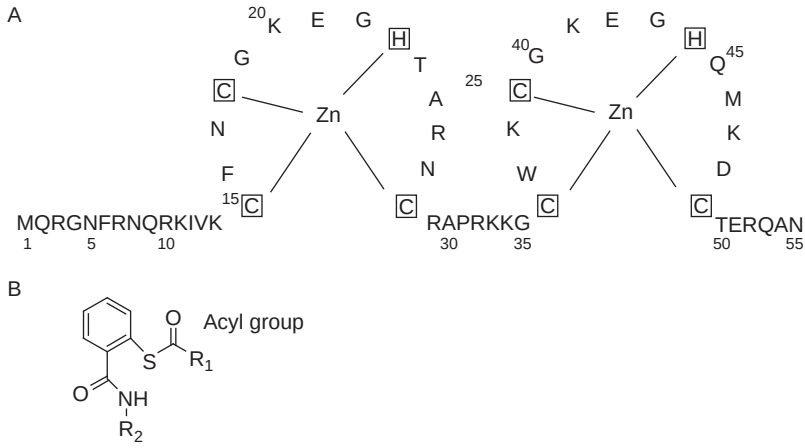


FIGURE 3 (A) Primary sequence of HIV-1 NCp7, showing zinc-coordination motif. Zinc-coordinating residues are boxed. (B) General SAMT template, with acyl group, R_1 (C = O).

residues are absolutely conserved across all known retroviruses, with additional amino acids surrounding the Cys and His residues displaying a similar conservation. It has been shown that altering any one of the zinc-chelating amino acids or any of a number of surrounding amino acids results in the production of noninfectious virus (Guo *et al.*, 2002; Ramboarina *et al.*, 1999; Tanchou *et al.*, 1998). NCp7 functions in the early phases of HIV replication by facilitating reverse transcription and mediating effects on integration and Tat transcription (Buckman *et al.*, 2003; Guo *et al.*, 2000; Hargittai *et al.*, 2004). Postintegration, the zinc fingers are important for autocatalysis of the Gag-Pol precursors to form functional protease (Turpin *et al.*, 1996; Zybarth and Carter, 1995), and then during formation of the new virus particle, the zinc fingers promote Gag assembly and interact with viral genomic RNA facilitating its incorporation into newly formed particles (Berkowitz *et al.*, 1993; Dawson and Yu, 1998; Shubsda *et al.*, 2002). In the mature virus particle, NCp7 coats the double-stranded HIV genomic RNA, acting to protect it from nucleases and degradation (Lapadat-Tapolsky *et al.*, 1993). The central role of the NCp7 protein in virus replication and its high conservation and presumably lower susceptibility to generation of resistant virus led to the hypothesis that if the NCp7 protein could be specifically targeted by small molecules, either in the virion or during one of its replication-critical functions, the result could be loss of virus infectivity and/or inhibition of replication.

Early drug discovery studies by Rice *et al.* (1993) were able to provide proof-of-concept that the NCp7 protein and its zinc fingers could be antiviral targets, and showed that disruption of these zinc fingers by removal of the coordinated zinc led to loss of infectivity, impairment of reverse transcription,

and Gag-precursor maturation. A variety of compounds have been shown to act covalently on one or several of the chelating cysteine residues through oxidative mechanisms (Loo *et al.*, 1996; Topol *et al.*, 2001). Notably among these were the 2,2′-dithiobis(benzamides) (DIBAs) (Rice *et al.*, 1993; Ryser *et al.*, 1994). Our research began with the task of optimizing the antiviral activity of the DIBA class of compounds. During the course of this investigation, we discovered the pyridinioalkanoyl thioesters (PATEs) (Turpin *et al.*, 1999), which replaced the disulfides with a thioester-based chemotype, leading ultimately to the design of a series of antiviral thioesters. The thioesters exhibited decreased *in vitro* cytotoxicity and higher specificity for the NCp7 target. These findings led to the development of a general class of thioesters, the N-substituted *S*-acyl-2-mercaptobenzamides (SAMTs), which are discussed below as candidate pluripotent, HIV-specific, virucidal microbicides. The SAMTs specifically target the cysteines of NCp7 (Jenkins *et al.*, 2005). The SAMTs use an *S*-acyl transfer mechanism in their reaction with NCp7, rather than an oxidative mechanism as described for previous NCp7 inhibitors (Jenkins *et al.*, 2005; Loo *et al.*, 1996; Topol *et al.*, 2001). Recently, we reported an additional step, a rapid intramolecular S to N acyl shift from the S-acyl cysteine to a nearby lysine side chain (Jenkins *et al.*, 2007).

VI. Characteristics of the SAMT Chemotype

Members of the SAMT chemotype (Fig. 3B) were investigated for a number of critical properties in terms of their candidacy as potential NCp7-targeted antivirals, including antiviral efficacy, cellular toxicity, and chemical properties. In general, the SAMT compounds inhibited HIV replication in both acutely and chronically HIV-infected *in vitro* cell models. These compounds demonstrated reduced *in vitro* cytotoxicity, improved aqueous solubility, nonsusceptibly to glutathione reduction, and retention of antiviral activity when exposed to human serum (Schito *et al.*, 2003; Srivastava *et al.*, 2004). These characteristics represented a significant improvement over the DIBAs in this inhibitor class. However, the SAMTs did not engender a significant improvement in antiviral potency over other NCp7 inhibitor chemotypes (50% inhibitory concentration 1–5 μM). Although their individual potency for inhibition of HIV replication was not improved, antiviral target specificity was retained, as demonstrated by virucidal activity in cell-free virions, ejection of Zn^{2+} from purified NCp7 protein, and the ability to cross-link NCp7 in virions and cells.

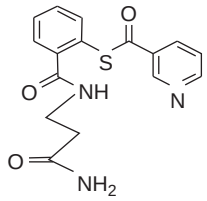
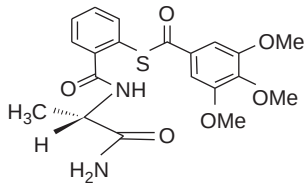
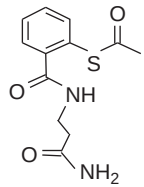
An early concern of targeting the NCp7 zinc finger was the potential for the inhibitors to interact with cellular zinc fingers that contain the same CCHC zinc-coordination motif, though with a different spacing between those residues, and with other zinc-coordination motifs (CCCC, CCHH, ring fingers, etc.) (Huang *et al.*, 1998; McGrath *et al.*, 2003), giving rise to

nonspecific toxicity. Although *in vitro* data suggested a strong rationale for antiviral activity and specificity of the SAMT inhibitors for NCp7 (Jenkins *et al.*, 2006), studies were needed to show that the SAMT retained these properties *in vivo*. To accomplish this, select members of the SAMT chemotype were tested for systemic reduction of virus expression and safety *in vivo* using an HIV-1 transgenic mouse model (Schito *et al.*, 2003). The early SAMT compounds were found to have no effect on immune cell cytokine production following delivery via osmotic pump for 10 days, while demonstrating a 2- to 3-log reduction in the infectivity of virus expressed from the spleen cells of transgenic mice. Expanded murine toxicity studies showed the maximum tolerated dosage (MTD) for intravenous treatment was between 160 and 320 mg/kg with an oral MTD>1 g/kg.

The next logical extension was to determine whether these small molecules were safe when administered to nonhuman primates. SAMT-19 (Table III) was delivered subdermally at a concentration of 2 mg/kg/day for 28 days by means of an osmotic pump to simian immunodeficiency virus (SIV) Delta/B670-infected cynomolgus monkeys (Schito *et al.*, 2006). The dosage selected represents the maximum deliverable SAMT-19 using the osmotic pump technology and does not represent the MTD for this model. Treatment began at either 7 or 14 days postinfection, and animals were monitored for viral load, infectivity of virus, and gag-specific T-cell responses. Safety was monitored by assessing serum chemistries (AST, ALT, urea, and BUN/creatine ratio). Although no significant or consistent reduction in plasma virus RNA copy was found, the time to peak viremia appeared to be delayed, and infectivity of SIV Delta/B670 recovered from plasma demonstrated a two- to threefold reduction in virus infectivity at 14–28 days postemplacement of the osmotic pump. More importantly, none of the animals demonstrated any significant alterations in lymphocyte numbers or serum chemistries or displayed unexpected clinical symptoms following 28 days of treatment. Thus, these results suggested that the SAMT chemotype can maintain antiviral activity *in vivo* without evidence of organism toxicity and that this chemotype was appropriate for further development.

These critical observations led us to focus on the SAMT chemotype and begin a program to optimize and identify the best possible candidate with properties that were compatible with a topical microbicide approach. We synthesized several hundred compounds, using SAMT template shown in Fig. 3B, and developed a multistep algorithm designed to characterize them for antiviral activity, cellular toxicity, and stability in buffers containing human serum (Srivastava *et al.*, 2004). This allowed us to define the essential elements of a pharmacophore structure bearing acyl groups that are uncharged in the physiological pH range. Anti-HIV activity was observed only for compounds where the sulfur atom was linked *ortho* to the benzoyl carbonyl site as shown in Fig. 3B (Srivastava *et al.*, 2004). The activity was relatively insensitive to acyl group structure and was optimal for the benzoyl

TABLE III Antiviral Activities (EC_{50} in μM) of Lead SAMTs

	19	*89*	*247*	*AZT*	*AT2*	*DS (mg/ml)*
CEM-SS/RF[a]	2.9	2.1	2.5	0.004	—	—
PBMC/B subtype[b]	1.1	0.02	ND	0.007	—	—
Mono/BaL[b]	5.0	<0.01	ND	0.01	—	—
PBMC/HIV-$2_{CDC310342}$[b]	0.5	5.9	ND	0.002	—	—
PBMC/SIV_{Mac251}[b]	1.7	4.7	ND	0.018	—	—
PBMC/MDR-769[b]	1.2	4.2	ND	0.6	—	—
Cobalt ejection[c]	35.9	15.4	29.6	—	—	—
Virucidal[d]	9.0	12.0	ND	—	2.2	—
Transmission:						
Syncytia[e]	0.6	0.9	ND	—	—	0.8
Replication[e]	0.08	0.1	ND	—	—	0.4

[a]Viral replication were determined on day 6 PI by measuring HIV cytoprotection using the tetrazolium dye derivative sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT).

[b]Viral replication were determined on day 7 PI by measuring supernatant RT activity or p24 antigen expression by ELISA.

[c]Cobalt was substituted for zinc to compare metal ion ejection rates of the compounds. Values represent milli-absorbance units.

[d]Reduction in infectivity of HIV-1 IIIB following a 4-h incubation with thioesters. Residual infectivity was determined in HeLa CD4 LTR β-gal cells by chemiluminescence.

[e]CEM-SS cells chronically infected with the SK-1 strain of HIV-1 were assessed for supernatant RT or syncytia formation.

Units are in μM unless stated otherwise.

AT2, alderthiol-2; DS, dextran sulfate (μg/ml); ND, not done; PBMC, peripheral blood mononuclear cell.

function linked to amino acid primary amides, namely, those of glycine, β-alanine, and D- or L-alanine. The ability to define specific and active pharmacophores demonstrated that antiviral activity and reactivity to the NCp7 zinc finger has a structural basis and was not the result of a nonspecific interaction with the cells or protein.

Because of the broad and critical involvement of the NCp7 protein in HIV-1 replication, NCp7 inhibitors can appear in functional assays to mimic the antiviral phenotypes associated with reverse transcriptase, protease, integrase, and/or maturation inhibitors, even though the inhibitors are

not directly blocking the same targets. Thus, in order to provide for the best possibility of selecting the optimal SAMT for advancement, a broad-based screening algorithm was developed. Initially, SAMT analogues were synthesized, purified, and then screened for antiviral activity, cellular toxicity, and structural stability in the presence of serum (Srivastava *et al.*, 2004). Antiviral activity was assessed using a combination of assays that looked at acute infection (NCp7 targets early in infection) and chronic infection (NCp7 targets postvirus integration). The standard HIV cytoprotection assay with CEM-SS cells with the laboratory adapted HIV-1 RF strain (Weislow *et al.*, 1989) was used for the acute infection assays. Measurement of virus using 166-murine HIV-1 transgenic spleen cells was used as a chronic/latent model of infection. The mouse transgenic spleen cells as a chronic/latent system for assessing antiviral reagents has been previously established with *env*-targeted toxins, as well as protease and reverse transcriptase inhibitors (Schito *et al.*, 2001, 2003). Both acute and chronic infection assays include a simultaneous determination of the test agents on cell viability, giving a dual indication of cytotoxicity and efficacy. Concordance for the ability of the SAMTs to inhibit both acute and chronic replication of HIV-1 was observed between the two assays in the vast majority of cases. The third arm of the algorithm consisted of assessing the stability of the SAMT in human pooled AB serum by HPLC. The decomposition of the thioester was measured over time to give an indication of its potential stability. The antiviral and toxicity data from the two assays along with the relative stability of the SAMT in human serum were then used to select seven compounds for further study.

In order to determine if the selected SAMT retained activities appropriate to an NCp7 inhibitor (broad range of action against other retroviruses, ejection of coordinated ions from the NCp7 zinc finger, and loss of infectivity of cell-free virions), a panel of profiling antiviral tests were performed (Table III). The three best of the seven lead compounds from the initial algorithm evaluations were tested against laboratory and primary strains of HIV-1, HIV-2, SIV, and a multidrug-resistant (MDR) clinical isolate in primary cells, as well as activity in monocytes/macrophages (Srivastava *et al.*, 2004). In addition, the compounds were tested for direct virucidal activity on cell-free virus and the ability to disrupt NCp7 protein structure by the release of coordinated cobalt from the NCp7 protein. Cobalt has been shown to be tetrahedrally coordinated in the CCHC motif of the NCp7 protein and can be used as a sensitive determination of ion coordination by the NCp7 zinc finger structure (Chen *et al.*, 2000). The lead compounds were all tested against clade-representative primary isolates of HIV-1, and shown to be equipotent on all HIV clades (unpublished data). SAMT-89 was found to synergistically inhibit virus replication without any indication of additional toxic effects in combination with both nonnucleoside and nucleoside reverse transcriptase, entry, protease inhibitors, and the NCp7 inhibitor, azodicarbonamide (unpublished data, Rice *et al.*, 1997). Thus, although our efforts did not

identify an NCp7 inhibitor with greatly enhanced potency over previous chemotypes, improvements in solubility and stability resulted in a significant advancement for NCp7 inhibitors as a class of candidate antivirals.

As noted in Table I, an important variable in the development of an ideal microbicide candidate is the ability to make a microbicide product that is synthetically compatible with the bulk manufacturing scale need to make the kilotons of product needed to allow a microbicide strategy to have a significant impact on the HIV pandemic. As part of the above efforts to generate a better "drugable" candidate SAMT, very early optimization of the synthetic pathways was also performed. The synthesis of SAMTs starts with the linkage of an amino acid amide to 2,2′-dithiosalicylic acid. These disulfide products are reduced to thiols and *S*-acylated using one of a variety of uncharged acid chloride reagents. The crude products can be crystallized from suitable solvents to increase purity with final characterization by NMR spectroscopy and thin-layer chromatography. Thus, synthesis of the SAMT compounds is straightforward, requiring only a few steps to generate pure material without need for specialized equipment, and should be readily adaptable to manufacture and scaling to cGMP production standards.

VII. Application of Thioesters for Microbicides

In order to determine if the thioester compounds were potential microbicide candidates, we initially tested the SAMTs for their ability to prevent cell-to-cell transmission of HIV-1. Table III shows that two of the lead SAMT analogs were able to potently prevent the transmission of HIV from chronically infected cells to uninfected cells. In this assay, chronically HIV-1 infected CEM-SS cells are mixed with uninfected cells and the ability of the infected cells to fuse with the uninfected cells via cell surface expressed gp120 and CD4 is measured via syncytia formation. Transmission of the virus and subsequent replication is measured by the ability of the newly fused cells to initiate new rounds of HIV-1 infection. SAMTs inhibited cell-to-cell HIV transmission, thereby providing initial evidence that these inhibitors could have utility as a topical microbicide.

A current benchmark for and a gateway in the development of potential topical microbicides has become determination of toxicity and antiviral activity in *ex vivo* vaginal tissue explant assays (Collins *et al.*, 2000; Greenhead *et al.*, 2000). Activity in this assay can be used to identify compatibility with vaginal tissues (toxicity) and potential efficacy. Assessment of SAMT-89 and 247 in cervical explant cultures showed that SAMTs were inhibitors of both the dissemination of virus from the explant by migrating cells and the infection of the explant tissue itself (Wallace *et al.*, 2006). More detailed experiments showed that the compounds' ability to inhibit virus dissemination was not due to SAMT altering virus binding to Dendritic Cell-Specific

ICAM3-Grabbing Nonintegrin (DC-SIGN). Interestingly, although the total HIV-1 p24 capsid protein produced by the explant tissues was not decreased following SAMT exposure, the infectivity of the released virus was compromised for the duration of the *ex vivo* culture (10 days). The 50% inhibitory concentrations (EC_{50}), for SAMTs in the explant model were around 0.01 mM, which is, on average, three to five times higher than that observed in *in vitro* cell-based virus protection assays. The SAMTs were also assessed for cytotoxic effects on vaginal and cervical epithelial cell lines, cervicovaginal explants, and artificial vaginal-ectocervical tissue matrixes (MatTek Corp.) and showed no significant toxicity at 0.5 mM. SAMTs also did not induce the production of the proinflammatory cytokines IL-1β and IL-8, further supporting that they were relatively nontoxic to the explant tissue. Finally the SAMTs were shown to retain antiviral activity in the presence of synthetic cervical mucus (G. Wallace, personal communication).

Next, we undertook to determine if the SAMT could prevent the transmission of SIV in a nonhuman primate model. However, before these studies could be undertaken, it had to be determined whether the thioester-based SAMT leads retained antiviral activity when resuspended with standard gel excipients. Initially, we determined the aqueous solubility of the lead SAMT analogs 89 and 247 and found that SAMT-247 was the more soluble of the two at 1 mg/ml. SAMT-247 was then resuspended by mixing in a hydroxyethylcellulose (HEC) formulation known as the "universal" placebo. This gel vehicle has been described by Tien *et al.* (2005) and is neither toxic nor protective in macaque SIV vaginal transmission and mouse HSV-2 vaginal challenge models and is being used as the placebo in several of the ongoing Phase II/IIB and III topical microbicide clinical trials. Figure 4 shows that when SAMT-247 was resuspended in the universal placebo vehicle at 1%, the antiviral activity of the resulting suspension was retained (EC_{50} SAMT 2.5 μM vs 1% suspension 10.8 μM). Antiviral activity was assessed both immediately and a week after resuspension without an apparent loss of activity. Since SAMT-89 showed less aqueous solubility than SAMT-247, we also assessed its ability to retain antiviral activity when incorporated in a film composed of inert hydroxypropylcellulose (HPC) and the microbicide candidate CAP. The dry film approach is more tolerant to solubility issues since the film, cast from an organic solvent system (Neurath *et al.*, 2003), acts as a suspension vehicle for the active ingredient(s), where incorporation can be independent of aqueous solubility. A film containing 1% SAMT-89 was made and found to retain antiviral activity and inhibit the replication of both R5 and X4 viruses on aqueous dispersion (A. R. Neurath and K. T. Jeang, personal communications, independent determinations of antiviral activity). This finding was supported by the observation that soluble CAP (dissolved at neutral pH) in combination with SAMT-89 reduced the replication of both X4 and R5 viruses in a synergistic manner (Jiang and Neurath, personal communications).

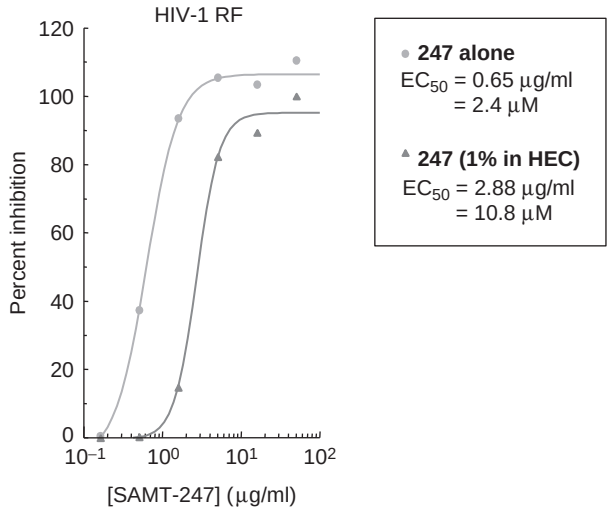


FIGURE 4 Formulated SAMTs retain antiviral activity to HIV-1 when tested in culture. SAMT-247 formulated in HEC was tested and compared to the compound alone by the standard cytoprotection assay. All dilutions were done in culture medium. EC_{50} is the median effective concentration required to induce a 50% effect.

The next logical extension of our efforts to determine whether SAMT are potential topical microbicide candidates was to determine if the SAMT could prevent vaginal transmission of SHIV in monkeys. Using the X4 and R5 dual infection model [CXCR4 (X4) $SHIV_{SF33A}$ and CCR5 (R5) $SHIV_{SF162P3}$] developed in Cecelia Cheng-Mayer's laboratory (Harouse *et al.*, 2003), we performed an initial pilot study. Six Depo-Provera pretreated rhesus macaques were pretreated with 2 ml of 1% SAMT-247 suspended in the HEC universal placebo gel 20 min prior to challenge with the mixed virus inoculum [150 $TCID_{50}$ (50% Tissue Culture Infective Dose) of each virus]. Five of six animals were protected from systemic infection, while the one infected animal expressed only the R5 SHIV (Cheng-Mayer, personnel communication). This is the first demonstration that a small molecule targeting the retroviral NCp7 can be used as a microbicide to successfully prevent infection.

VIII. Target Specificity of the SAMT NCp7 Inhibitors

The failure of the Col-1492 trial of Nonoxynol-9 resulted in the rethinking of the role of agents which act to inactivate virus, and it led to the virtual elimination of detergents from the development pipeline. One rationale for this concern and a more conservative approach to virucidal agents as microbicides is based on the simple question of how to inactivate the virus without also nonspecifically targeting other cellular structures. One major concern during

the initial development of the NCp7 inhibitors was reaction with other cellular zinc fingers. Examination of the cytotoxicity data accompanying the initial publication of 3-nitrosobenzamide (NOBA) (Rice *et al.*, 1992) and the DIBAs shows that these initial chemotypes were cytotoxic to both transformed cell lines and primary cells. During the development of the SAMT chemotype, we identified that the SAMT tended to be more toxic on transformed cell lines than primary cells. As originally shown by Rice *et al.* (1992), some of the NCp7 inhibitor chemotypes are more toxic to transformed cells since the parental chemotypes may interact with the CCHC zinc finger of the poly(ADP-ribose) polymerase-1 (PARP) enzyme leading to apoptosis. Later, Wang *et al.* (2004) showed that it is possible to identify inhibitors targeting the zinc fingers of the estrogen receptor in estrogen-dependent breast cancer cells and tamoxifen-resistant cells (Wang *et al.*, 2006). Therefore, as we developed the SAMTs, it became critical that we not only determine the precise mechanism of the zinc ejection from the NCp7 protein by these inhibitors, but also develop a rationale for why there is apparent selectivity both *in vitro* and *in vivo*, as evidenced by lack of cellular and organismal toxicity.

The SAMTs, as well as other NCp7 inhibitor chemotypes, have been shown to mediate a variety of covalent modifications of cysteine residues in the retroviral zinc finger domains of NCp7 (Basrur *et al.*, 2000; Jenkins *et al.*, 2005; Loo *et al.*, 1996; Topol *et al.*, 2001). These covalent modifications result in the formation of inter-NCp7 disulfide bonds that are indicative of NCp7 inhibitor interaction with the NCp7 protein. We have shown that exposure of both cell-free virus and HIV-infected cells to the SAMT thioester chemotype results in formation of adducts to the cysteine side chain sulfur atoms via an acyl migration from the thioester (unpublished data). These adducts result in distortion of the structure of the zinc finger and disruption of NCp7 function (Fig. 5), leading to ejection of the coordinated metal ion and loss of virus infectivity. In order to better understand this interaction, we have employed spectrophotometric methods to study the release of Co^{2+} from cobalt-refolded NCp7 following treatment with SAMT compounds (Table III). These studies have proven to be an expedient and sensitive method to gauge the metal ejection properties of the thioester congeners. NMR spectroscopy experiments were used to further investigate the interaction of the lead compounds with the NCp7 zinc fingers (Jenkins *et al.*, 2005). It was shown that metal loss occurred predominantly from the C-terminal zinc finger, with metal ejection occurring in the N-terminal finger only after loss of the zinc-coordination fold of the C-terminal finger. This result highlights the specificity of the SAMT compounds—though the two zinc fingers have identical zinc-coordination motifs and folds, and even a high degree of sequence similarity, the SAMT reaction is preferentially initiated in the C-terminal zinc finger. Mass spectral analysis identified acyl adducts on the cysteine residues in the C-terminal finger, demonstrating the covalent modification of the cysteine residues by the

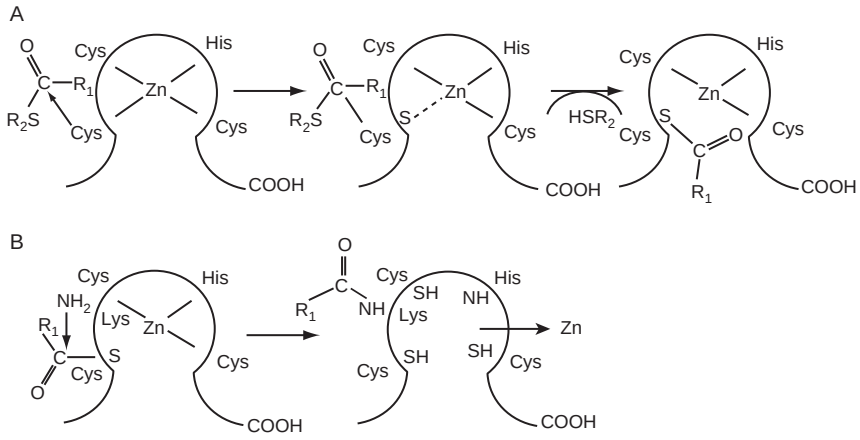


FIGURE 5 Proposed acyl transfer mechanism of action of the SAMT thioester compounds. (A) In the primary intermolecular transacylation, the sulfur of Cys36 makes a nucleophilic attack on the carbonyl carbon of the thioester. This attack results in transfer of the acyl group onto the cysteine sulfur. (B) In the secondary intramolecular transfer, the amine of a proximal lysine residue initiates an S to N transfer from the cysteine to the lysine. This second transfer disrupts zinc coordination, leading to loss of the zinc finger structure and subsequent loss of NCp7-mediated functions

thioester compounds (Basrur *et al.*, 2000; Jenkins *et al.*, 2005). Previous studies on other compounds that interact with NCp7 have also shown this specificity of interaction with the C-terminal zinc finger (Chertova *et al.*, 1998; Loo *et al.*, 1996; Topol *et al.*, 2001). Furthermore, computational analysis of the protein packing and electrostatic screening of zinc finger domains suggested that the C-terminal zinc finger was among the most exposed and available for reaction (Maynard and Covell, 2001).

Based on our data, we have proposed the following model for attack of the NCp7 zinc finger by the SAMT inhibitors (Fig. 5). The reaction mechanism begins when a cysteine sulfur undergoes a nucleophilic attack on the carbonyl carbon of the thioester compound. This nucleophilic attack results in covalent modification of the cysteine sulfur (Fig. 5A), followed by rapid transfer of the acyl adduct to a nearby lysine amino group (Fig. 5B) and loss of the coordinated zinc ion. The transfer of an adduct to a proximal lysine residue results in this group being more stably, and essentially irreversibly, bound. This process further distorts the overall fold of the zinc finger, leading to collapse of the fold of the C-terminal zinc finger. As the two zinc finger domains are not entirely independent, the loss of structure in the C-terminal finger eventually leads to the loss of fold in the N-terminal domain as well. Thus, SAMTs specifically interact with the C-terminal zinc finger of NCp7, causing the protein to lose both structure and function. Additionally, it was demonstrated that incubation of NCp7 with the SAMT compounds blocked the ability of the protein to specifically bind to viral

RNA sequences (Jenkins *et al.*, 2005). Thus, the SAMT compounds not only disrupt the structure of the protein, but also its function. The C-terminal amino acid residues under attack appear to be highly conserved, critical to the functionality of the NCp7 protein, and mechanistically are the attack point for the SAMT, thus suggesting that engendering resistance would not be easy. Efforts within the laboratory using standard methodologies employing serial culture in increasing concentration of zinc finger inhibitor all of which have failed to generate specific resistance to any of the published SAMT chemotypes (unpublished data), demonstrating that the NCp7 inhibitors may not have the problem of escape by resistance often associated with currently used antiviral therapies.

Interestingly, both the thiols and the thioesters have equally potent antiviral activity in the cell-based assays in the presence or absence of high concentrations of human serum (up to 50%) (Ott, personal communication). However, in analytical experiments, such as those described above, involving recombinant NCp7 in cell-free buffers, chelated cobalt or zinc ions were not ejected by the thiol form of the inhibitor, only by the intact thioester. For example, as observed in NMR spectroscopy experiments, the thiol, under anaerobic conditions, produced little effect on the fold of recombinant NCp7, unlike the clear disruption of fold observed with thioester compounds (Jenkins *et al.*, 2005). We have hypothesized that in the intracellular environment, a thiol component, either produced by interaction with serum or cell esterases or released by reaction with NCp7, may be re-acylated intracellularly to form a new thioester. We further hypothesized that acyl coenzyme A (acyl CoA) was a potential mediator of the re-acylation. Recently, we have tested this hypothesis by demonstrating that when the thiol was pre-incubated with acetyl CoA and then mixed with NCp7, a significant increase in metal ion ejection was observed over thiol or acetyl CoA alone (Jenkins *et al.*, 2005). This result suggests that thiol components can be rapidly re-acylated by acyl-CoA or other acyl transfer factors to regenerate the virucidal entity. Experiments have suggested that the individual thiol molecule may be recycled through these processes numerous times, resulting in the repeated creation of a reactive thioester compound.

IX. Conclusions

Topical microbicides have the potential to significantly impact the HIV/AIDS pandemic by providing vulnerable populations with a self-administered method to prevent HIV infection. Although we are still awaiting the identification of a safe, effective, and acceptable topical microbicide for general use, there is strong evidence that an effective topical microbicide could potentially save millions of lives. In order to meet the promise of topical microbicides, not only does clinical efficacy, safety, and acceptance

need to be shown, but new microbicides and targets need to be developed to provide for the next generations of microbicide strategies. The identification of a safe and effective HIV-specific, virucidal microbicide has not been a priority for development, as evidenced by their limited representation in the microbicide pipeline since safety concerns were raised for the nonspecific, detergent, virucide Nonoxynol-9. The optimal virucidal microbicide would be one that targets not only cell-free but also cell-associated virus, acting as a pluripotent, HIV-specific virucide, capable of targeting multiple steps in the HIV replication cycle. This potentially has been achieved through the discovery and development of the thioester-based, *S*-acyl-2-mercaptobenzamide compounds targeting the HIV-1 NCp7 nucleocapsid zinc finger. Experimental evidence shows that these inhibitors interact with a highly conserved protein sequence in a covalent manner to prevent the replication of both cell-free (virucidal) and cell-associated virus by irreversibly ejecting the coordinated zinc ion from the protein, thus altering its structure. This inhibition holds for all clades and MDR HIV isolates tested. Loss of NCp7 structure/function leads to inhibition of HIV replication and infectivity due to interruption of the many activities of the nucleocapsid zinc fingers in virus replication. Thioester-based compounds have long been considered as poorly "drugable" and zinc fingers considered as potentially nonspecific targets. However, our lead compounds, developed as NCp7 inhibitors, have addressed and overcome these issues both *in vitro* and *in vivo*. The lead NCp7 inhibitors retain functionality in high concentrations of serum, artificial mucin, and potential formulation excipients. *In vivo* delivery subdermally via an osmotic pump, and in a gel vehicle, has provided preliminary evidence that SAMTs can retain anti-HIV potency and remain safe under *in vivo* conditions. Experimental approaches, designed to establish the thioester-based SAMTs as topical microbicide candidates, demonstrated efficacy and safety in *ex vivo* cervicovaginal tissue explants and protection in nonhuman primate vaginal transmission models. Although these data are encouraging, the development of NCp7 zinc finger inhibitors as a viable candidate topical microbicide still requires further study. The evidence above suggests that NCp7 inhibitors, specifically the SAMT lead compounds 89 and 247, are strong microbicide candidates which can add significantly to the current microbicide pipeline by providing a first-in-class, pluripotent, HIV-specific virucide.

X. Addendum

At the time of writing, cellulose sulfate was in Phase III clinical trials in Benin, India, Nigeria, South Africa, and Uganda. On January 31, 2007, the trial in Africa was halted by its sponsor CONRAD, when a routine safety review indentified preliminary results that suggested cellulose sulfate could lead to an increased risk of HIV infection in women who used the microbicide

gel. A second Phase III trial of cellulose sulfate sponsored by Family Health International was also halted as a precautionary measure. At the time of this proof, although the final results have not been published, an oral presentation at the 2007 International AIDS Society meeting in Sydney Australia (July 26, 2007) identified a non-statistical trend toward harm (Risk ratio 1.6) with 25 infections in the treatment arm and 16 in the placebo with 1428 randomized participants following an intent-to-treat analysis.

References

Basrur, V., Song, Y., Mazur, S. J., Higashimoto, Y., Turpin, J. A., Rice, W. G., Inman, J. K., and Appella, E. (2000). Inactivation of HIV-1 nucleocapsid protein p7 by pyridinioalkanoyl thioesters. Characterization of reaction products and proposed mechanism of action. *J. Biol. Chem.* **275**, 14890–14897.

Berg, J. M. (1986). Potential metal-binding domains in nucleic acid binding proteins. *Science* **232**, 485–487.

Berkowitz, R. D., Luban, J., and Goff, S. P. (1993). Specific binding of human immunodeficiency virus type 1 *gag* polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. *J. Virol.* **67**, 7190–7200.

Boadi, T., Schneider, E., Chung, S., Tsai, L., Gettie, A., Ratterree, M., Blanchard, J., Neurath, A. R., and Cheng-Mayer, C. (2005). Cellulose acetate 1,2-benzenedicarboxylate protects against challenge with pathogenic X4 and R5 simian/human immunodeficiency virus. *AIDS* **19**, 1587–1594.

Buckman, J. S., Bosche, W. J., and Gorelick, R. J. (2003). Human immunodeficiency virus type 1 nucleocapsid Zn^{2+} fingers are required for efficient reverse transcription, initial integration processes, and protection of newly synthesized viral DNA. *J. Virol.* **77**, 1469–1480.

Catalone, B. J., Kish-Catalone, T. M., Budgeon, L. R., Neely, E. B., Ferguson, M., Krebs, F. C., Howett, M. K., Labib, M., Rando, R., and Wigdahl, B. (2004). Mouse model of cervicovaginal toxicity and inflammation for preclinical evaluation of topical vaginal microbicides. *Antimicrob. Agents Chemother.* **48**, 1837–1847.

Centers for Disease Control and Prevention (2000). CDC statement on study results of product containing nonoxynol-9. *JAMA* **284**, 1376.

Chan, D. J. (2005). Factors affecting sexual transmission of HIV-1: Current evidence and implications for prevention. *Curr. HIV Res.* **3**, 223–241.

Chen, X., Chu, M., and Giedroc, D. P. (2000). Spectroscopic characterization of Co(II)-, Ni(II)-, and Cd(II)-substituted wild-type and non-native retroviral-type zinc finger peptides. *J. Biol. Inorg. Chem.* **5**, 93–101.

Chertova, E. N., Kane, B. P., McGrath, C., Johnson, D. G., Sowder, R. C., 2nd, Arthur, L. O., and Henderson, L. E. (1998). Probing the topography of HIV-1 nucleocapsid protein with the alkylating agent N-ethylmaleimide. *Biochemistry* **37**, 17890–17897.

Cohen, M. S., and Pilcher, C. D. (2005). Amplified HIV transmission and new approaches to HIV prevention. *J. Infect. Dis.* **191**, 1391–1393.

Cole, A. M. (2006). Innate host defense of human vaginal and cervical mucosae. *Curr. Top. Microbiol. Immunol.* **306**, 199–230.

Collins, K. B., Patterson, B. K., Naus, G. J., Landers, D. V., and Gupta, P. (2000). Development of an *in vitro* organ culture model to study transmission of HIV-1 in the female genital tract. *Nat. Med.* **6**, 475–479.

Cone, R. A., Hoen, T., Wong, X., Abusuwwa, R., Anderson, D. J., and Moench, T. R. (2006). Vaginal microbicides: Detecting toxicities *in vivo* that paradoxically increase pathogen transmission. *BMC Infect. Dis.* **6**, 90.

Coombs, R. W., Speck, C. E., Hughes, J. P., Lee, W., Sampoleo, R., Ross, S. O., Dragavon, J., Peterson, G., Hooton, T. M., Collier, A. C., Corey, L., Koutsky, L., *et al.* (1998). Association between culturable human immunodeficiency virus type 1 (HIV-1) in semen and HIV-1 RNA levels in semen and blood: Evidence for compartmentalization of HIV-1 between semen and blood. *J. Infect. Dis.* **177**, 320–330.

Covey, S. N. (1986). Amino acid sequence homology in gag region of reverse transcribing elements and the coat protein gene of cauliflower mosaic virus. *Nucleic Acids Res.* **14**, 623–633.

D'Cruz, O. J., and Uckun, F. M. (2004). Clinical development of microbicides for the prevention of HIV infection. *Curr. Pharm. Des.* **10**, 315–336.

Dawson, L., and Yu, X. F. (1998). The role of nucleocapsid of HIV-1 in virus assembly. *Virology* **251**, 141–157.

Dezzutti, C. S., James, V. N., Ramos, A., Sullivan, S. T., Siddig, A., Bush, T. J., Grohskopf, L. A., Paxton, L., Subbarao, S., and Hart, C. E. (2004). *In vitro* comparison of topical microbicides for prevention of human immunodeficiency virus type 1 transmission. *Antimicrob. Agents Chemother.* **48**, 3834–3844.

Dhawan, D., and Mayer, K. H. (2006). Microbicides to prevent HIV transmission: Overcoming obstacles to chemical barrier protection. *J. Infect. Dis.* **193**, 36–44.

Doncel, G. F., Chandra, N., and Fichorova, R. N. (2004). Preclinical assessment of the proinflammatory potential of microbicide candidates. *J. Acquir. Immune Defic. Syndr.* **37**(Suppl. 3), S174–S180.

Foss, A. M., Vickerman, P. T., Heise, L., and Watts, C. H. (2003). Shifts in condom use following microbicide introduction: Should we be concerned. *AIDS* **17**, 1227–1237.

Frank, I., and Pope, M. (2002). The enigma of dendritic cell-immunodeficiency virus interplay. *Curr. Mol. Med.* **2**, 229–248.

Garg, S., Anderson, R. A., Chany, C. J., II, Waller, D. P., Diao, X. H., Vermani, K., and Zaneveld, L. J. D. (2001). Properties of a new acid-buffering bioadhesive vaginal formulation (ACIDFORM). *Contraception* **64**, 67–75.

Gayle, H. D. (2006). AIDS anniversaries in 2006 mark the time to deliver. *Lancet* **368**, 425–427.

Greenhead, P., Hayes, P., Watts, P. S., Laing, K. G., Griffin, G. E., and Shattock, R. J. (2000). Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. *J. Virol.* **74**, 5577–5586.

Guo, J., Wu, T., Anderson, J., Kane, B. F., Johnson, D. G., Gorelick, R. J., Henderson, L. E., and Levin, J. G. (2000). Zinc finger structures in the human immunodeficiency virus type 1 nucleocapsid protein facilitate efficient minus- and plus-strand transfer. *J. Virol.* **74**, 8980–8988.

Guo, J., Wu, T., Kane, B. F., Johnson, D. G., Henderson, L. E., Gorelick, R. J., and Levin, J. G. (2002). Subtle alterations of the native zinc finger structures have dramatic effects on the nucleic acid chaperone activity of human immunodeficiency virus type 1 nucleocapsid protein. *J. Virol.* **76**, 4370–4378.

Gupta, P., Mellors, J., Kingsley, L., Riddler, S., Mandaleshwar, K. S., Schreiber, S., Cronin, M., and Rinaldo, C. R. (1997). High viral load in semen of human immunodeficiency virus type 1-infected men at all stages of disease and its reduction by therapy with protease and nonnucleoside reverse transcriptase inhibitors. *J. Virol.* **71**, 6271–6275.

Haase, A. T. (2005). Perils at mucosal front lines for HIV and SIV and their hosts. *Nat. Rev. Immunol.* **5**, 783–792.

Hargittai, M. R., Gorelick, R. J., Rouzina, I., and Musier-Forsyth, K. (2004). Mechanistic insights into the kinetics of HIV-1 nucleocapsid protein-facilitated tRNA annealing to the primer binding site. *J. Mol. Biol.* **337**, 951–968.

Harouse, J. M., Buckner, C., Gettie, A., Fuller, R., Bohm, R., Blanchard, J., and Cheng-Mayer, C. (2003). CD8 + T cell-mediated CXC chemokine receptor 4-simian/human immunodeficiency virus suppression in dually infected rhesus macaques. *Proc. Natl. Acad. Sci. USA* **100**, 10977–10982.

Huang, M., Maynard, A., Turpin, J. A., Graham, L., Janini, G. M., Covell, D. G., and Rice, W. G. (1998). Anti-HIV agents that selectively target retroviral nucleocapsid protein zinc fingers without affecting cellular zinc finger proteins. *J. Med. Chem.* **41**, 1371–1381.

Jenkins, L. M. M., Byrd, J. C., Hara, T., Srivastava, P., Mazur, S., Stahl, S. J., Inman, J. K., Appella, E., Omichinski, J. G., and Legault, P. (2005). Studies on the inactivation of the HIV-1 nucleocapsid protein NCp7 with 2-mercaptobenzamide thioesters. *J. Med. Chem.* **48**, 2847–2858.

Jenkins, L. M. M., Durell, S. R., Maynard, A. T., Stahl, S. J., Inman, J. K., Appella, E., Legault, P., and Omichinski, J. G. (2006). Comparison of the specificity of interaction of cellular and viral zinc-binding domains with 2-mercaptobenzamide thioesters. *J. Am. Chem. Soc.* **128**, 11964–11976.

Jenkins, L. M. M., Hara, T., Durell, S. R., Hayashi, R., Inman, J. K., Piquemal, J.-P., Gresh, N., and Appella, E. (2007). Specificity of acyl transfer from 2-mercaptobenzamide thioesters to the HIV-1 nucleocapsid protein. *J. Am. Chem. Soc.* **129**, 11067–11078.

Karmon, E., Potts, M., and Getz, W. M. (2003). Microbicides and HIV: Help or hindrance? *J. Acquir. Immune Defic. Syndr.* **34**, 71–75.

Klasse, P. J., Shattock, R. J., and Moore, J. P. (2006). Which topical microbicides for blocking HIV-1 transmission will work in the real world? *PLoS Med.* **3**, e351.

Lapadat-Tapolsky, M., De Rocquigny, H., Van Gent, D., Roques, B., Plasterk, R., and Darlix, J.-L. (1993). Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. *Nucleic Acids Res.* **21**, 831–839.

Lard-Whiteford, S. L., Matecka, D., O'Rear, J. J., Yuen, I. S., Litterst, C., Reichelderfer, P., and Microbicides, I. W. G. O. (2004). Recommendations for the nonclinical development of topical microbicides for prevention of HIV transmission: An update. *J. Acquir. Immune Defic. Syndr.* **36**, 541–552.

Lederman, M. M., Offord, R. E., and Hartley, O. (2006). Microbicides and other topical strategies to prevent vaginal transmission of HIV. *Nat. Rev. Immunol.* **6**, 371–382.

Lee, B. M., De Guzman, R. N., Turner, B. G., Tjandra, N., and Summers, M. F. (1998). Dynamical behavior of the HIV-1 nucleocapsid protein. *J. Mol. Biol.* **279**, 633–649.

Loo, J. A., Holler, T. P., Sanchez, J., Gogliotti, R., Maloney, L., and Reily, M. D. (1996). Biophysical characterization of zinc ejection from HIV nucleocapsid protein by anti-HIV 2,2′-dithiobis[benzamides] and benzisothiazolones. *J. Med. Chem.* **39**, 4313–4320.

Lu, H., Zhao, Q., Wallace, G., Liu, S., He, Y., Shattock, R., Neurath, A. R., and Jiang, S. (2006). Cellulose acetate 1,2-benzenedicarboxylate inhibits infection by cell-free and cell-associated primary HIV-1 isolates. *AIDS Res. Hum. Retrov.* **22**, 411–418.

Maynard, A. T., and Covell, D. G. (2001). Reactivity of zinc finger cores: Analysis of protein packing and electrostatic screening. *J. Am. Chem. Soc.* **123**, 1047–1058.

McGrath, C. F., Buckman, J. S., Gagliardi, T. D., Bosche, W. J., Coren, L. V., and Gorelick, R. J. (2003). Human cellular nucleic acid-binding protein Zn2 + fingers support replication of human immunodeficiency virus type 1 when they are substituted in the nucleocapsid protein. *J. Virol.* **77**, 8524–8531.

MDS Working Groups (2006). The Microbicide Development Strategy.

Miller, C. J., and Shattock, R. J. (2003). Target cells in vaginal HIV transmission. *Microbes Infect.* **5**, 59–67.

Miller, C. J., Li, Q., Abel, K., Kim, E. Y., Ma, Z. M., Wietgrefe, S., La Franco-Scheuch, L., Compton, L., Duan, L., Shore, M. D., Zupancic, M., Busch, M., *et al.* (2005). Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J. Virol.* **79**, 9217–9227.

Motakis, D., and Parniak, M. A. (2002). A tight-binding mode of inhibition is essential for anti-human immunodeficiency virus type 1 virucidal activity of nonnucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **46**, 1851–1856.

Neurath, A. R., Strick, N., Li, Y.-Y., and Debnath, A. K. (2001). Cellulose acetate phthalate, a common pharmaceutical excipient, inactivates HIV-1 and blocks the coreceptor binding site on the virus envelope glycoprotein gp120. *BMC Infect. Dis.* **1**, 17.

Neurath, A. R., Strick, N., Jiang, S., Li, Y.-Y., and Debnath, A. K. (2002). Anti-HIV-1 activity of cellulose acetate phthalate: Synergy with soluble CD4 and induction of dead-end gp41 six-helix bundles. *BMC Infect. Dis.* **2**, 6.

Neurath, A. R., Strick, N., and Li, Y.-Y. (2003). Water dispersible microbicidal cellulose acetate phthalate film. *BMC Infect. Dis.* **3**, 27.

O'Connor, T. J., Kinchington, D., Kangro, H. O., and Jeffries, D. J. (1995). The activity of candidate virucidal agents, low pH and genital secretions against HIV-1 *in vitro*. *Int. J. STD AIDS* **6**, 267–272.

Ongradi, J., Ceccherini-Nelli, L., Pistello, M., Specter, S., and Bendinelli, M. (1990). Acid sensitivity of cell-free and cell-associated HIV-1: Clinical implications. *AIDS Res. Hum. Retrov.* **6**, 1433–1436.

Patton, D. L., Sweeney, Y. C., Tsai, C. C., and Hillier, S. L. (2004). *Macaca fascicularis* vs. *Macaca nemestrina* as a model for topical microbicide safety studies. *J. Med. Primatol.* **33**, 105–108.

Pereira, C. F., and Paridaen, J. T. M. L. (2004). Anti-HIV drug development—an overview. *Curr. Pharm. Des.* **10**, 4005–4037.

Pilcher, C. D., Tien, H. C., Eron, J. J., Jr., Vernazza, P. L., Leu, S. Y., Stewart, P. W., Goh, L. E., Cohen, M. S., Study, Q., and Consortium, D.-U.-E. A. H. (2004). Brief but efficient: Acute HIV infection and the sexual transmission of HIV. *J. Infect. Dis.* **189**, 1785–1792.

Pope, M., and Haase, A. T. (2003). Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. *Nat. Med.* **9**, 847–852.

Quayle, A. J., Xu, C., Mayer, K. H., and Anderson, D. J. (1997). T lymphocytes and macrophages but not motile spermatozoa, are a significant source of human immunodeficiency virus in semen. *J. Infect. Dis* **176**, 960–968.

Ramboarina, S., Morellet, N., Fournie-Zaluski, M. C., and Roques, B. P. (1999). Structural investigation on the requirement of CCHH zinc finger type in nucleocapsid protein of human immunodeficiency virus 1. *Biochemistry* **38**, 9600–9607.

Rice, W. G., Hillyer, C. D., Harten, B., Schaeffer, C. A., Dorminy, M., Lackey, D. A., Kirsten, E., Mendeleyev, J., Buki, K. G., Hakam, A., and Kun, E. (1992). Induction of endonuclease-mediated apoptosis in tumor cells by C-nitroso-substituted ligands of poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA* **89**, 7703–7707.

Rice, W. G., Schaeffer, C. A., Harten, B., Villinger, F., South, T. L., Summers, M. F., Henderson, L. E., Bess, J. W., Jr., Arthur, L. O., McDougal, J. S., Orloff, S. L., Mendeleyev, J., *et al.* (1993). Inhibition of HIV-1 infectivity by zinc-ejecting aromatic *C*-nitroso compounds. *Nature* **361**, 473–475.

Rice, W. G., Turpin, J. A., Huang, M., Clanton, D., Buckheit, R. W., Jr., Covell, D. G., Wallqvist, A., McDonnell, N. B., DeGuzman, R. N., Summers, M. F., Zalkow, L., Bader, J. P., *et al.* (1997). Azodicarbonamide inhibits HIV-1 replication by targeting the nucleocapsid protein. *Nat. Med.* **3**, 341–345.

Ryser, H. J. J., Levy, E. M., Mandel, R., and DiSciullo, G. J. (1994). Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. *Proc. Natl. Acad. Sci. USA* **91**, 4559–4563.

Schito, M. L., Kennedy, P. E., Kowal, R., Berger, E. A., and Sher, A. (2001). A human immunodeficiency virus transgenic mouse model for assessing interventions that block microbial-induced proviral expression. *J. Infect. Dis.* **183**, 1592–1600.

Schito, M. L., Goel, A., Song, Y., Inman, J. K., Fattah, R. J., Rice, W. G., Turpin, J. A., Sher, A., and Appella, E. (2003). *In vivo* antiviral activity of novel human immunodeficiency virus type 1 nucleocapsid p7 zinc finger inhibitors in a transgenic murine model. *AIDS Res. Hum. Retrov.* **19**, 91–101.

Schito, M. L., Soloff, A. C., Slovitz, D., Trichel, A., Inman, J. K., Appella, E., Turpin, J. A., and Barratt-Boyes, S. M. (2006). Preclinical evaluation of a zinc finger inhibitor targeting lentivirus nucleocapsid protein in SIV-infected monkeys. *Curr. HIV Res.* **4**, 379–386.

Shubsda, M. F., Paoletti, A. C., Hudson, B. S., and Borer, P. N. (2002). Affinities of packaging domain loops in HIV-1 RNA for the nucleocapsid protein. *Biochemistry* **41**, 5276–5282.

Smith, R. J., Bodine, E. N., Wilson, D. P., and Blower, S. M. (2005). Evaluating the potential impact of vaginal microbicides to reduce the risk of acquiring HIV in female sex workers. *AIDS* **19**, 413–421.

South, T. L., Blake, P. R., Hare, D. R., and Summers, M. F. (1991). C-terminal retroviral-type zinc finger domain from the HIV-1 nucleocapsid protein is structurally similar to the N-terminal zinc finger domain. *Biochemistry* **30**, 6342–6349.

Srivastava, P., Schito, M., Fattah, R. J., Hara, T., Hartman, T., Buckheit, R. W., Jr., Turpin, J. A., Inman, J. K., and Appella, E. (2004). Optimization of unique, uncharged thioesters as inhibitors of HIV replication. *Bioorgan. Med. Chem.* **12**, 6437–6450.

Stein, Z. A. (1990). HIV prevention: The need for methods women can use. *Am. J. Public Health* **80**, 460–462.

Stone, A. (2002). Microbicides: A new approach to preventing HIV and other sexually transmitted infections. *Nat. Rev. Drug Discov.* **1**, 977–985.

Stone, A. B. (2004). HIV topical microbicides: The current development strategy is fully justified. *Am. J. Public Health* **94**, 1845–1846.

Tanchou, V., Decimo, D., Pechoux, C., Lener, D., Rogemond, V., Berthoux, L., Ottmann, M., and Darlix, J. L. (1998). Role of the N-terminal zinc finger of human immunodeficiency virus type 1 nucleocapsid protein in virus structure and replication. *J. Virol.* **72**, 4442–4447.

Tien, D., Schnaare, R. L., Kang, F., Cohl, G., McCormick, T. J., Moench, T. R., Doncel, G., Watson, K., Buckheit, R. W., Lewis, M. G., Schwartz, J., Douville, K., *et al.* (2005). *In vitro* and *in vivo* characterization of a potential universal placebo designed for use in vaginal microbicide clinical trials. *AIDS Res. Hum. Retrov.* **10**, 845–853.

Topol, I. A., Nemukhin, A. V., Dobrogorskaya, Y. I., and Burt, S. K. (2001). Interactions of azodicarbonamide (ADA) species with the model zinc finger site: Theoretical support of the zinc finger domain destruction in the HIV-1 nucleocapsid protein (NCp7) by ADA. *J. Phys. Chem. B* **105**, 11341–11350.

Turpin, J. A. (2003). The next generation of HIV/AIDS drugs: Novel and developmental anti HIV drugs and targets. *Expert Rev. Anti. Infect. Ther.* **1**, 97–128.

Turpin, J. A., Terpening, S. J., Schaeffer, C. A., Yu, G., Glover, C. J., Felsted, R. L., Sausville, E. A., and Rice, W. G. (1996). Inhibitors of human immunodeficiency virus type 1 zinc fingers prevent normal processing of gag precursors and result in the release of noninfectious virus particles. *J. Virol.* **70**, 6180–6189.

Turpin, J. A., Song, Y., Inman, J. K., Huang, M., Wallqvist, A., Maynard, A., Covell, D. G., Rice, W. G., and Appella, E. (1999). Synthesis and biological properties of novel pyridinioalkanoyl thiolesters (PATE) as anti-HIV-1 agents that target the viral nucleocapsid protein zinc fingers. *J. Med. Chem.* **42**, 67–86.

UNAIDS (2006). AIDS Epidemic Update: Special Report on HIV/AIDS: December 2006.

van de Wijgert, J., Fullem, A., Kelly, C., Mehendale, S., Rugpao, S., Kumwenda, N., Chirenje, Z., Joshi, S., Taha, T., Padian, N., Bollinger, R., and Nelson, K. (2001). Phase 1 trial of the topical microbicide BufferGel: Safety results from four international sites. *J. Acquir. Immune Defic. Syndr.* **26**, 21–27.

Veazey, R. S., Klasse, P. J., Schader, S. M., Hu, Q., Ketas, T. J., Lu, M., Marx, P. A., Dufour, J., Colonno, R. J., Shattock, R. J., Springer, M. S., and Moore, J. P. (2005). Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. *Nature* **438**, 99–102.

Vickerman, P., Watts, C., Delany, S., Alary, M., Rees, H., and Heise, L. (2006). The importance of context: Model projections on how microbicide impact could be affected by the underlying epidemiologic and behavioral situation in 2 African settings. *Sex. Transm. Dis.* **33**, 397–405.

Wallace, G., Schito, M. L., and Appella, E. (2006). HIV-1 nucleocapsid zinc finger inhibitors (zfi's) impede trans infection in cellular and explant models. *Microbicides* April 23–26, 2006: Abstract OA25.

Wang, L. H., Yang, X. Y., Zhang, X., Mihalic, K., Fan, Y. X., Xiao, W., Howard, O. M. Z., Appella, E., Maynard, A. T., and Farrar, W. L. (2004). Suppression of breast cancer by chemical modulation of vulnerable zinc fingers in estrogen receptor. *Nat. Med.* **10**, 40–47.

Wang, L. H., Yang, X. Y., Zhang, X., An, P., Kim, H.-J., Huang, J., Clarke, R., Osborne, C. K., Inman, J. K., Appella, E., and Farrar, W. L. (2006). Disruption of estrogen receptor DNA binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer. *Cancer Cell* **10**, 487–499.

Weber, J., Desai, K., Darbyshire, J., and Programme, M. D. (2005). The development of vaginal microbicides for the prevention of HIV transmission. *PLoS Med.* **2**, 142.

Weislow, O. S., Kiser, R., Fine, D. L., Bader, J., Shoemaker, R. H., and Boyd, M. R. (1989). New soluble-formazan assay for HIV-1 cytopathic effects—application to high-flux screening of synthetic and natural-products for AIDS-antiviral activity. *J. Natl. Cancer Inst.* **81**, 577–586.

Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K. A., Reimann, K. A., Reinhart, T. A., Rogan, M., Cavert, W., Miller, C. J., Veazey, R. S., Notermans, D., *et al.* (1999). Sexual transmission and propagation of SIV and HIV in resting and activated CD4 + T cells. *Science* **286**, 1353–1357.

Zybarth, G., and Carter, C. (1995). Domains upstream of the protease (PR) in human immunodeficiency virus type 1 Gag-Pol influence PR autoprocessing. *J. Virol.* **69**, 3878–3884.

Miguel E. Quiñones-Mateu[*,†,‡], **Dawn M. Moore-Dudley**[§], **Oyebisi Jegede**[†], **Jan Weber**[*], **and Eric J. Arts**[‡,§,‖]

[*]HIV-1 Program, Diagnostic Hybrids, Athens, Ohio 45701

[†]Department of Cellular and Molecular Biology, Kent State University, Kent, Ohio 44242

[‡]Center for AIDS Research, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106

[§]Department of Molecular and Microbiology, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106

[‖]Division of Infectious Diseases, BRB 1029, Case Western Reserve University, Cleveland, Ohio 44106

Viral Drug Resistance and Fitness

I. Chapter Overview

The common feature of all antiretroviral drugs (ARVs), regardless of class or target, is the selection and emergence of resistant HIV-1 variants. Resistance is typically conferred by discrete and well-characterized mutation(s) for drugs that target the reverse transcriptase (RT inhibitors or RTIs), protease (PR inhibitors or PIs), and integrase enzymes (IN inhibitors or INIs) of the virus, but the pattern of mutations to entry inhibitors (EIs) appears more complex and dependent on sequence context in the envelope (*env*) gene. A replicative fitness cost is associated with nearly all RTI, PI, INI resistance mutations when the respective drug is absent and suggests that

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56009-6

these mutations are favored by the enzyme. In gp41, mutations conferring resistance to fusion inhibitors may be related to an immediate fitness cost but resistance can also be associated with increased fusion kinetics. The same appears to be true with the new class of CCR5 agonists/antagonists. Many resistant CCR5-tropic HIV-1 variants have enhanced entry efficiency-related increased coreceptor affinities, rather than the typical impairment in function associated with the ARVs that target enzymatic function. This chapter will highlight recent findings related to HIV-1 drug resistance and fitness.

II. Introduction

Antiretroviral therapy is a form of extrinsic pressure applied against the *Human immunodeficiency virus 1* (HIV-1) that reduces replication but also selects for variants with reduced sensitivity to this pressure. These HIV-1 variants are typically present in the infecting virus population ("swarm" or "quasispecies") prior to treatment but are maintained at low frequency due to their low fitness. Fitness is defined by the replicative capacity of the virus in a given environment but it is important to note that this "environment" is in constant flux. For example, an HIV-1 clone harboring an M184V mutation in the reverse transcriptase (RT) coding region is likely present at very low frequency in the intrapatient HIV-1 population due to its low fitness. Upon administration of 3TC (or lamivudine), this 3TC-resistant, M184V HIV-1 variant is immediately selected in the population and can be considered the most "fit" clone in this environment. Several studies have suggested the presence of a lower viral load in a 3TC treated patient harboring M184V HIV-1 variant as compared to an untreated patient harboring "wild-type" HIV-1 (Deval *et al.*, 2004; White *et al.*, 2002). The M184V HIV-1 is basically insensitive to 3TC so this reduction in virus load is attributed to reduced replicative capacity of the virus. This hypothesis is obviously too simplistic and undoubtedly other factors such as immune response may accentuate the reduced viral load. In addition, the M184V mutation is at the extreme in terms of debilitating effects on the virus and the level of resistance to 3TC. In this chapter, we have examined the resistance to each class of antiviral drug and impact of these resistance mutations on replicative fitness in the absence of drug. In summary, the mutations conferring resistance to EIs appear to have the most impact on the function of the Env glycoproteins in virus replication whereas resistance to PIs and RTIs is conferred by mutations that decrease the fitness of these enzymatic functions. To date, few studies have explored how the viruses evolve and compensate throughout the genome during selection at a single gene level by ARV. Hopefully, future studies will address if a decrease in replicative fitness conferred by a specific drug resistance mutation (e.g., M184V) extends to

the entire virus or if the M184V virus rapidly compensates for this defect. In addition, this review has focused on emerging studies with EIs in relation to drug resistance and fitness. Several recent reviews have carefully addressed the fitness impact of resistance to PIs and RTIs.

A. Entry Inhibitors

1. Resistance to CCR5 and CXCR4 Antagonists

a. Binding Inhibitors There are few studies on resistance to EIs considering only T-20 (enfuvirtide or ENF) has been utilized for anti-HIV treatment. Thus, most analyses are based on selection of *in vitro* drug resistance while passing virus in the presence of increasing doses of the inhibitor. Although these studies have led to important insights on the functional mechanisms of these inhibitors, it is important to note that *in vitro* resistance and selection of specific mutations must be compared with *in vivo* selection through the use of these EIs in treatment. Unlike the consistent emergence of specific drug resistance mutations in the RT and PR genes of relatively low genetic diversity, HIV-1 *env* diversity is so great that it is possible that different evolutionary pathway will be required for the emergence of resistance to EIs. Thus, resistance to EIs must be examined in an *in vivo* context once these drugs, such as maraviroc, are approved for such use.

As described earlier, several studies have shown patterns of drug resistance mutations conferring resistance to EIs. These mutations were either part of the "wild-type" sequence and confer "intrinsic" resistance (Torre *et al*., 2000) or were selected through serial virus passing with escalating doses of drug (Kuhmann *et al*., 2004; Marozsan *et al*., 2005a; Trkola *et al*., 2002; Westby *et al*., 2007). Trkola *et al.* (2002) were the first to describe the emergence of an HIV-1 escape variant to the binding inhibitor AD101 that was 20,000-fold more resistant than the wild-type virus. Sequence analysis of the *env* gene consensus sequence identified 22 amino acid changes during the 19 passages of the CCR5 tropic HIV-1 isolate in the presence of the drug. Four of these 22 amino acid changes appeared in the V3 region of Env and were both necessary and sufficient for AD101 resistance (Kuhmann *et al*., 2004). These four amino acids include K305R, A316V, and G321E, all in the presence of H308P. In Fig. 1, the mutations selected under AD101 pressure are identified in the gp120 crystal structure. Interestingly, the mutation at position 308 appeared dependent on sequence context and on regions outside of V3. This was the first evidence that *env* genetic diversity could define an evolutionary "resistance" pathway for a given virus. This AD101 escape variant utilized low levels of CCR5 on target cells better than its parental virus and did not escape by switching tropism to CXCR4 usage. This AD101 escape variant was also partially cross-resistant to the highly related drug SCH-C but showed no cross-resistance to PA14 (a monoclonal antibody that targets CCR5) or RANTES.

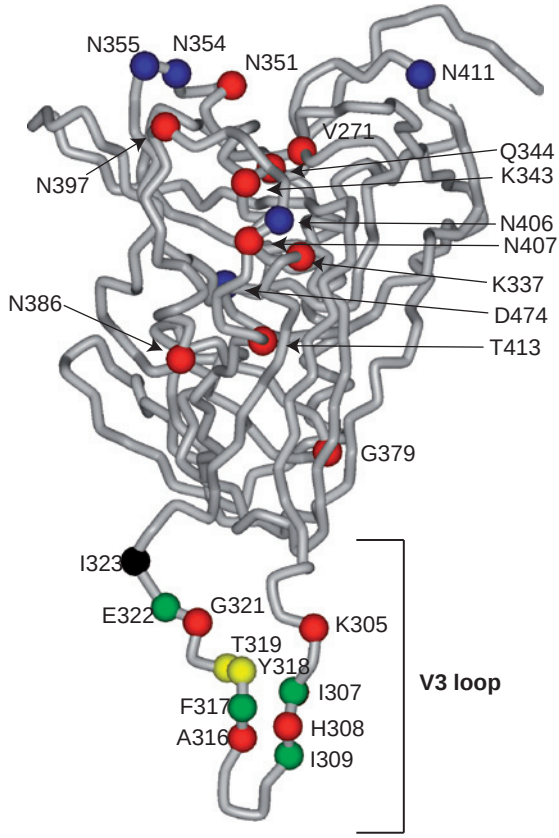


FIGURE 1 Location of amino acid substitutions in the Env gp120 crystal structure selected by *in vitro* passage with CCR5 antagonists. Unlike the primary resistance mutations directly associated with RTIs, PIs, and INIs, HIV-1 appears to follow different evolutionary pathways to resistance dependent on the *env* sequence and CCR5 or CXCR4 antagonist. The amino acids in the HIV-1 Env gp120 structure (Huang *et al.*, 2005) selected or associated with resistance to AD101 are shown in red, SCH-D—blue, Tak-779—green, RANTES—yellow, and maraviroc—black. As described in the text, studies have not fully confirmed which mutations are responsible for primary resistance and which are secondary or compensatory.

The same group who selected for AD101 resistance has independently selected for resistance to SCH-D (SCH-417690) by passing two distinct viruses in the presence of this drug (Marozsan *et al.*, 2005a). The SCH-D escape mutants harbored V3 *env* sequences identical to the parental or mutated a single codon at position 308. Analyses of a larger *env* fragment revealed a total of 17 amino acid substitutions starting in the C1 domain of gp120 through the ectodomain of gp41 (Fig. 1). The specific mutations conferring resistance have not yet been fully identified but it is obvious

that complex linkage of mutations was involved in SCH-D escape. Again, coreceptor switching was not a mechanism of escape. Rather, one of the SCH-D escape variants was capable of utilizing CCR5 in its SCH-D-bound conformation (Pugach *et al.*, 2007) and did not show a classical resistance profile (i.e., an increase in the concentration required for 50% inhibition or IC_{50} value). This SCH-D-resistant clone also displayed a unique cross-resistance profile to AD101, SCH-C, CMPD 167, RANTES, PSC-RANTES, and AOP-RANTES. However, cross-resistance, particularly to PSC-RANTES, could have been due to significant levels of SCH-D remaining with the virus supernatant (because of passaging in the presence of high doses of drug). Cross-resistance of an SCH-D-resistant clone to AD101 was, however, confirmed using "washed" virus (Pugach *et al.*, 2007). In addition, the SCH-D-resistant clone did not display reduced sensitivity to fusion inhibitors including T-20, T-1249, and the attachment inhibitor CD4-IgG2, as well as to PIs and RTIs (Marozsan *et al.*, 2005a). Finally, Pugach *et al.* (2007) studied the effect of SCH-D and PRO 140 CCR5 antagonism on chemokine release and CCR5 expression levels in peripheral blood mononuclear cells (PBMCs). Levels of MIP-1β and RANTES, but not MIP-1α, were significantly increased in PBMCs from seven different donors exposed to SCH-D or PRO-140. CCR5 expression was also increased in the presence of SCH-D. These results indicate that the effects of CCR5 antagonists on immune response must be considered along with the ability of the drug to inhibit HIV-1 infection.

Drug resistance mutations have also been characterized for the small molecule CCR5 antagonist TAK-779. In one study, a library of V3 loop mutations within HIV-$1_{JR\text{-}FL}$ was created containing 10 amino acid substitutions. The mutations were randomly placed into 31 CCR5-tropic HIV-1 strains for a possible 27,648 different V3 sequences (Yusa *et al.*, 2005). This library was passaged in the presence of increasing concentrations of TAK-779 to yield a population of viruses 15-fold more resistant to TAK-779 than viruses passaged without TAK-779. Introduction of five amino acid substitutions (I304V, H305N, I306M, F312L, and E317D) (Fig. 1) into the parental HIV-$1_{JR\text{-}FL}$ resulted in a virus with 16-fold resistance to TAK779. Although TAK-779 is not considered a suitable drug candidate, viruses harboring these mutations must be analyzed for cross-resistance to TAK-652 and other EIs. Any mutation that may alter the sensitivity to one EI may have general effects on virus-binding affinity and entry and thus, may influence sensitivity to another EI. Drug resistance was also studied for TAK-652 following serial virus passage for more than one year with increasing doses of TAK-652 (Baba *et al.*, 2007). The resistant virus was 200,000-fold more resistant to TAK-652 than the parental wild-type virus and was also cross-resistant to TAK-779, but not to TAK-220, which binds to different regions of CCR5. When the Env was sequenced from the highly resistant isolate, 12 amino acids changes were found in the C2, V3, V4, and C4

regions of gp120 as well as 5 mutations in the gp41 region (Fig. 1). The exact mutations associated with resistance have not been analyzed but the resistance mechanism was again not associated with a switch to CXCR4-tropism.

Drug resistance was also assessed for the R5 inhibitor maraviroc, again by passaging HIV-1 isolates in increasing doses of the inhibitor (Westby *et al.*, 2007). Maraviroc-resistant virus did not emerge from serial passaging of the laboratory strain, HIV-1_{BAL}. However, when primary HIV-1 viruses were assessed, three out of six viruses developed resistance (two patient isolates and HIV-1_{SF162}). The two patient isolates developed resistance through different evolutionary pathways and with different combination of mutations (Fig. 1). One resistant virus developed two amino acid changes in the V3 region at positions 316 and 323, as well as several mutations in other regions of the *env*. However, clones harboring only these V3 mutations were not resistant to maraviroc (Westby *et al.*, 2007). The resistant virus containing the two V3 amino acid mutations lost the 316 mutation, but kept the 323 mutation in the absence of drug. The other resistant patient isolate had a QAI deletion in the V3 crown, which was associated with resistance but quickly reverted to the wild-type V3 crown in the absence of maraviroc. The HIV-1_{SF-162} resistant virus developed the ability to utilize CXCR4; however, this was regardless of whether or not the virus was passaged in the presence of maraviroc, indicating that this selection occurred as a result of passaging in peripheral blood lymphocytes (PBLs). The maraviroc-resistant viruses were not cross-resistant to SCH-C and T-20 (Westby *et al.*, 2007). Finally, it appeared that one of the clones from the resistant virus population containing the 316 and 323 mutations was able to utilize CCR5 in the maraviroc-bound conformation. Briefly, a maraviroc-resistant/aplaviroc-sensitive clone became resistant to aplaviroc if maraviroc was added first to the cultures. Aplaviroc does not compete with maraviroc for binding to CCR5 but both block HIV-1 binding by a similar uncompetitive mechanism. Since this maraviroc-resistant virus can bind to the CCR5 complexed to maraviroc, it can also occlude the aplaviroc-binding site on CCR5.

Selection for HIV-1 resistance to a CCR5 antagonist or agonist requires longer passage times and more passages than previous resistance selection experiments involving PIs or RTIs. The more "difficult" selection for EI resistance is attributable to the necessity of using primary CCR5-tropic HIV-1 isolates as opposed to the CXCR4-tropic laboratory strains, typically employed for resistance selection studies with PIs or RTIs. On average, R5 HIV-1 replicates significantly slower than X4 HIV-1 strains but this may be more related to affinity of the virus for the two coreceptors and increase CXCR4 receptor density on cell lines (Quinones-Mateu and Arts, 2002b). Another confounding factor for the selection of resistance to CCR5 antagonist is related to a threshold of inhibition, that is, the inability of most R5 antagonist to block 100% of virus entry (Kuhmann *et al.*, 2004; Marozsan *et al.*, 2005a; Trkola *et al.*, 2002; Westby *et al.*, 2007). Finally, some HIV-1

EIs (e.g., RANTES and SDF-1α derivatives), acting through CCR5 or CXCR4, maintain partial agonist activities and mediate a signaling cascade in the cell (Chang *et al.*, 2002; Gordon *et al.*, 1999; Marozsan *et al.*, 2001; Trkola *et al.*, 1999). This signaling can then stimulate the replication of the HIV-1 that escaped the initial inhibitory activity of the drug.

All of these factors have likely contributed to the failure of selecting R5 HIV-1 isolates resistant to RANTES derivatives such as PSC- or AOP-RANTES, despite repeated attempts by multiple research groups. Nonetheless, primary HIV-1 isolates display variable susceptibility to EIs and in the case of the RANTES derivatives, a 50-fold increase in IC_{50} values has been labeled "intrinsic resistance" (Torre *et al.*, 2000). Amino acid substitutions at position 318 and 319 in the V3 loop have been identified as sites associated with intrinsic resistance to AOP-RANTES (Fig. 1) (Torre *et al.*, 2000). The "resistant" amino acids (Y318 and A/T319) are found at higher frequency in the wild-type HIV-1 population than the "sensitive" and rare amino acid polymorphisms (R318 and R319), suggesting a possible *in vivo* selection by RANTES or neutralizing antibodies (Lobritz *et al.*, 2007). In addition, virus labeled as "intrinsically resistant" to PSC-RANTES showed a general cross-resistance to all EIs, but wild-type sensitivity to PIs and RTIs. In the next section, this "intrinsic resistance" will be compared to increased entry kinetics and higher replicative fitness.

In general, most drug resistance mutations associated with CCR5 antagonists inhibitors have been identified in the V3 loop of gp120. This is not surprising considering their location could be directly related to involvement of this V3 loop in binding to the second extracellular loop of CCR5. Nonetheless, several studies have identified mutations outside of the V3 loop that still confer resistance to CCR5 antagonists. The connection between these mutations, resistance to EIs, and potential resistance mechanisms is unclear especially considering that these mutations are spread throughout the gp120 and gp41 glycoproteins.

b. Attachment Inhibitors Drug resistance has been described for three different attachment inhibitors. Cyanovirin N (CV-N) targets the high mannose glycans in gp120 as does concanavalin A (ConA), but the ConA has a lower specificity for high mannose glycans than CV-N (Witvrouw *et al.*, 2005). Escalating the dose of these lectins in the presence of replicating $HIV\text{-}1_{NL4\text{-}3}$, a CXCR4-tropic virus, for 20 passages resulted in resistant viruses. Virus resistant to CV-N harbored two mutations in the gp120 region (N302K and N418S) as well as a deletion from position 364 to 376. The virus resistant to ConA showed four amino acid changes including N204K, S261F, N302K, and T311I. What is remarkable about many of these mutations is that they simply eliminate multiple N-glycan sites on gp120 necessary for the addition of mannose glycans and subsequent extrinsic binding by ConA or CV-N.

Resistance to BMS-806, an inhibitor that binds to gp120 and prevents attachment to CD4, emerged after passaging HIV-1_{NL4-3} and HIV-1_{LAI} in increasing concentrations of drug. Multiple mutations in the *env* gene were selected including I595F, M475I, M434I/V, K655E, R350K, S440R, V68A, D185N, and M426L (Lin *et al.*, 2003) (Fig. 1). Only M434I and M475I were identified in both BMS-806-resistant NL4-3 and LAI viruses. Further analysis of these mutations in a subsequent study showed that the M426, M434, and M475 mutations were linked to significant decreases in BMS-806 sensitivity while the other mutations were only compensatory (Guo *et al.*, 2003). These mutations were also analyzed in terms of their influence on CD4-gp120 binding to a cavity in gp120 and extension into a water-filled channel responsible for disrupting BMS-806-gp120 complex (Madani *et al.*, 2004). Drug resistance to BMS-806 was directly associated with mutations that alter the drug-binding site in gp120 but do not affect CD4 interactions. Nevertheless, mutations in other regions of the Env, namely in the gp120 bridging sheet, the inner domain, and receptor-binding sites of Env were also responsible for up to a 40-fold resistance to BMS-806 (Madani *et al.*, 2004).

2. *Resistance to Fusion Inhibitors*

ENF is the only EI approved by the FDA for the treatment of HIV-infected individuals. Clinical resistance to ENF has been well characterized and studied *in vivo*. Resistance mutations to ENF generally appear in the drug targeted region of the gp41 (heptad repeat 1 or HR1). Early *in vitro* studies indicated that CCR5-tropic viruses were more resistant than CXCR4-tropic viruses to ENF and T-649, a fusion inhibitor directed against the gp41 HR2 region (Derdeyn *et al.*, 2000, 2001; Reeves *et al.*, 2003). All of the mutations conferring resistance to fusion inhibitors are shown in the crystal structure models of the gp41 six alpha helix bundle (Fig. 2). Considering that HIV-1 tropism is defined by the V3 region of gp120, there are likely regions outside of the ENF-binding domain that can also mediate susceptibility to ENF. However, phase III clinical trials have indicated that coreceptor tropism did not statistically correlate with virologic response to ENF treatment *in vivo*, even though the CXCR4-tropic viruses had a 2.2-fold higher 50% inhibitory concentration (IC_{50}) than the CCR5-tropic viruses (Melby *et al.*, 2006).

The first three amino acid changes associated with resistance to ENF were located in the gp41 HR1 domain in what is now known as the GIV motif. These amino acid changes are located in the gp41 positions 36–38 and were first identified following *in vitro* serial passage of the virus in the presence of increasing ENF concentrations (Derdeyn *et al.*, 2000, 2001; Rimsky *et al.*, 1998) (Fig. 2). T-649 was also sensitive to mutations in the GIV motif of HIV-1 isolates. It has been shown that amino acid position 36 of this motif plays a dominant role in determining the fusogenic activity of a given HIV-1 Env (Kinomoto *et al.*, 2005). An aspartic acid at position 36,

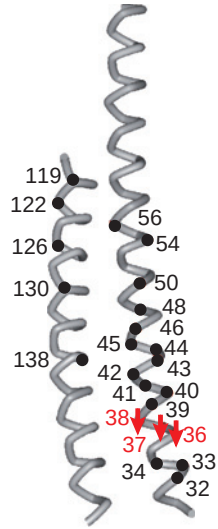


FIGURE 2 Location of amino acid substitutions in the Env gp41 crystal structure selected during treatment or *in vitro* passage with fusion inhibitors. The crystal structure depicts two facing alpha helices from the HR1 and HR2 domains within the six alpha helix bundle (Chan *et al*., 1997). The mutations associated with resistance to ENF, C34, and N44* peptides are labeled. A black circle identifies mutations selected under drug pressure but without a tested effect on replicative fitness. A gray arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.

rather than glycine, is associated with increased fusion kinetics, leading to resistance to ENF. By the end of phase I and II clinical trials in HIV-1-infected patients, the region conferring resistance to ENF was expanded to positions 36–45 of gp41 (Lu *et al*., 2004; Wei *et al*., 2002) and provided further evidence that *in vitro* mutations may be predictive of *in vivo* drug resistance mutations to ENF.

In addition to this GIV motif in gp41, regions in gp120, namely those that influence coreceptor usage, also impacted the virus sensitivity to T-649 (Derdeyn *et al*., 2001; Rimsky *et al*., 1998). Reeves *et al*. first described that regions outside of this GIV motif influenced virus sensitivity to ENF. Affinity for the coreceptor greatly influenced drug sensitivity to ENF by altering fusion kinetics in relation to receptor density (Reeves *et al*., 2003). This difference in fusion kinetics could also account for the variable sensitivity of primary HIV-1 isolates to ENF and almost all other EIs (Labrosse *et al*., 2003). Fusion kinetics and sensitivity to ENF can be influenced by a variety of factors including ICAM-1 incorporation into the viral membrane carried over from the infected cell (Beausejour and Tremblay, 2004). More ICAM-1 incorporation leads to greater resistance to ENF, faster fusion kinetics, and/or an increase in receptor affinity.

To determine the impact of ENF resistance mutations on cross-resistance to other EIs, mutations in the GIV motif were introduced into a CCR5- or CXCR4-tropic virus backbone. These viruses were more sensitive to neutralizing antibodies but showed minimal cross-resistance to other EIs including sCD4, BMS-806, AMD3100, TAK-779, or T-1249 (Reeves *et al.*, 2005). The relationship between entry kinetics and general sensitivity to EIs remains a subject of debate but two resistance mechanisms appear possible: (1) the virus may evolve to increase entry efficiency as general mechanism for reduced sensitivity to EIs, and (2) specific mutations may be associated with the lack of drug binding or drug exclusion. These mechanisms can emerge independently or possibly act synergistically to confer resistance.

N44* and C34 are peptide inhibitors that target the six-helix bundle of gp41 by mimicking a region of the N-peptide (Desmezieres *et al.*, 2005; Nameki *et al.*, 2005). These peptides were produced specifically to study the mechanism of escape developed by C- and N-peptide fusion inhibitors. Following *in vitro* selection with N44*, the escaped virus variant could replicate in the presence of 1200 nM of N44* while the parental was inhibited by 400 nM of the drug (Desmezieres *et al.*, 2005). Two specific mutations (Q577R and E648K) were associated with low-level resistance to N44* and weak cross-resistance to ENF and C34 (Fig. 2). Surprisingly, these N44* drug resistance mutations were shown to increase six-helix bundle stability and as result decreased the binding capacity of N44*. *In vitro* selection of C34 resistance resulted in a combination of linked mutations in gp41 (Nameki *et al.*, 2005) (Fig. 2). A careful *in vitro* mapping analyses of these mutations revealed that I37K (in gp41) was the amino acid most associated with a drug resistance phenotype. However, the inclusion of D36G, N126K, L204I, and the V4 gp120 deletion (ΔFNSTW) in the I37K backbone was responsible for high-level C34 resistance (83-fold) and cross-resistance to ENF (64-fold) (Nameki *et al.*, 2005). These findings suggest that rate and stability of the six alpha helix formation may be responsible for a general resistance mechanism for peptides that target either the HR1 or HR2 domains of gp41.

3. Fitness of Viruses Resistant to EIs

Fitness is defined as the replication capacity of a virus in a defined environment (i.e., *ex vivo* in tissue culture or *in vivo* in the presence of drugs or an intact immune system). *Ex vivo* fitness (a measure in viral competitions in PBMCs) of an HIV-1 isolate is a correlate of disease progression and drug resistance is typically associated with a decrease in fitness (Quinones-Mateu *et al.*, 2000). Fitness can often help determine how and why a specific virus emerges out of a population, or perhaps even why a specific subtype may be emerging in the epidemic as a whole (Arien *et al.*, 2007). Because most EIs are still on the horizon as possible ARVs, the impact of EI drug resistance mutations on fitness have been studied only on a few

occasions and primarily in relation to ENF resistance. The following will review the possible fitness costs related to EI resistance.

In general, the *env* gene of HIV-1 has a significant impact on the fitness of a virus (Ball *et al*., 2003; Marozsan *et al*., 2005b; Rangel *et al*., 2003). The ability of a virus to initially bind to CCR5 and enter a cell plays a dominant role in determining replication efficiency throughout the virus life cycle (Marozsan *et al*., 2005b). Beyond this initial entry step, it is likely that other steps of HIV-1 replication play a role in replicative fitness but entry remains the bottleneck to "weed out" less fit variants. This concept of viral competition also occurs at the level of a virus quasispecies within a patient. A quasispecies is a population of genetically distinct HIV-1 isolates produced due to the low fidelity and high mutation rates of HIV-1 RT (Quinones-Mateu and Arts, 2002b, 2006). This population of viruses constantly competes with each other for survival within the environment of that patient. This environment is not static but changes over time with decline in immune response or introduction of extrinsic drug pressure. Since virus fitness is defined by the environment, the dominance of specific virus clones is dependent on the strongest selective pressure at a given time. On the basis of the notion that the entry process is dominant in determining HIV-1 fitness, it can be extrapolated that sensitivity to drugs that impact this process, that is, EIs, are also likely to impact the fitness of a virus. Several groups have now suggested increased HIV-1 binding to CCR5 in association with resistance to CCR5 antagonists or agonists. In addition, many of the mutations associated with resistance to SCH-C, AD101, or maraviroc may increase the efficiency of host cell entry. To date, there is no published data on the replicative fitness of HIV-1 variants resistant to EIs other than ENF. Preliminary data would suggest that EI resistance may not always be associated with decreased replicative fitness and in a few examples, increased entry efficiency and fitness may be the actual mechanism of resistance (Lobritz *et al*., 2007).

Multiple studies have linked ENF resistance mutations to reductions in HIV-1 fitness. The best characterized drug resistance mutations to ENF are changes in the amino acids 36–38 of gp41 that is, the GIV motif. Lu *et al*. (2004) introduced these mutations into the HIV-1_{NL4-3} backbone and analyzed the *ex vivo* replication capacity of these viruses both in the presence and absence of ENF. They found that in the presence of the drug, the ENF-resistant mutants were the most fit. In the absence of ENF, the wild-type virus could outcompete the resistant viruses in *ex vivo* competitions. The most fit virus in the presence of ENF was the least fit virus in the absence of the EI, which is the typical observation with most drug-resistant HIV-1 isolates. These specific amino acid substitutions conferring ENF resistance were analyzed in the absence of any other secondary sequence changes and in the absence of the genetic context giving rise to this mutational profile. It is highly likely that *in vivo*, a virus that develops resistance mutations will

also select for mutations in other regions of Env to compensate for any fitness costs. Nevertheless, this study provided the proof-of-concept that EI resistance mutations will indeed detrimentally affect the fitness of a virus. This observation was further supported by another study indicating that mutations in the GIV motif could reduce virus replication in PBMC cultures (Reeves *et al*., 2005).

A study by Labrosse *et al*. (2006) has clearly indicated that sequence context and secondary mutations in the *env* genes can lessen the impact of the primary ENF resistance mutations. Pseudotyped viruses derived from the *env* gene of patients before, during, and after ENF treatment suggested that efficiency of host cell entry was maintained in the *env* gene throughout the course of treatment and after the emergence of resistance. These results suggest that the emergence of drug resistance *in vivo* may not be associated with a reduction in fitness of a virus. Again, the impact of drug resistance on *in vivo* fitness is likely different than the impact of drug resistance on *in vitro* fitness due to HIV-1 sequence context, the role of host response, and varying drug pressures (Deeks *et al*., 2007; Labrosse *et al*., 2006; Menzo *et al*., 2004).

Intrinsic resistance to various EIs has been described but for the most part, these HIV-1 isolates are considered "wild type" and obviously survive competition within the patient's virus quasispecies. Neumann *et al*. (2005) focused on intrinsic drug resistance to ENF by cloning the *env* genes of these patients into the HIV-1_{NL4-3} backbone and determining the impact of intrinsic resistance on replicative fitness. This study found that rather than a loss in fitness, the *env* genes from the ENF insensitive virus had similar fitness. One of these three *env* genes from the "intrinsic resistance" class actually encoded for viral glycoproteins with increased fitness. Considering that these viruses did not evolve under drug pressure, it is quite possible that any mutations that conferred ENF resistance and reduced fitness could be rapidly compensated by other mutations in *env*.

The impact of drug resistance mutations on fitness provides some rationale for maintaining a patient on a failing regimen due to some virologic benefit of reduced viral fitness. A recently published study by Deeks *et al*. (2007) described the benefit of continuing ENF salvage treatment in patients failing an ENF-containing regimen. It is speculated that despite failure on various ARV treatment regimens, patients may benefit by maintaining drug-resistant viruses that are generally associated with a reduction of fitness and perhaps slower disease progression. Given the high cost of ENF, the difficulty in administration of this drug, and adverse side effects, this study attempted to determine the benefit of keeping patients on ENF. Removal of ENF in patients failing their treatment regimen resulted in a small but minor increase in plasma HIV-1 RNA levels and a slight but insignificant decrease in $CD4^+$ T-cell counts. The efficiency of the patient-derived *env* genes to mediate entry was examined before treatment interruption, and

then on multiple time points after the treatment interruption (Deeks *et al.*, 2007). In the absence of ENF, *env* pseudotyped viruses were more efficient at entry following ENF removal but lost their resistance to ENF. This data suggest that increased replication capacity of these viruses may be associated with a loss of ENF resistance or rather reversion to wild type. This study utilized only single-cycle assays and did not perform head-to-head competitions to determine fitness of the viruses with *env* genes derived before and after treatment interruption.

As indicated earlier, a CCR5 antagonist, maraviroc has been recently approved for treatment but there have no published preclinical studies describing the impact of maraviroc resistance mutations on replicative fitness. There have been reports of the lack of a fitness impact or even fitness increases associated with resistance to SCH-D (Anastassopoulou *et al.*, 2006). These studies have yet to be confirmed and compared to the fitness impacts associated with resistance to vicriviroc, aplaviroc, TAK-779, or the various RANTES derivatives. In regard to the RANTES derivatives, Lobritz *et al.* (2007) assessed the fitness of natural V3 polymorphisms associated with intrinsic resistance to RANTES derivates (e.g., PSC-RANTES) as well as most other classes of EIs (e.g., ENF and TAK-779). There was a direct and significant correlation between the sensitivity to each EI (IC_{50} value) and replicative fitness (as determined by dual virus competitions). In other words, more frequent V3 polymorphisms (318Y and 319A/T) were associated with increased replicative fitness and reduced sensitivity (or resistance) to EIs whereas the less frequent, less fit polymorphisms (318R and 319R) were also hypersusceptible to EIs (Lobritz *et al.*, 2007). These findings suggest that HIV-1 isolates with increased replicative fitness related to increased entry efficiency may prevent or reduce drug binding and confer resistance.

More in-depth studies are required to resolve the potential impact of EI resistance on replicative fitness. However, it is quite likely that the mechanism of increased entry efficiency may be associated with increased fitness and a general resistance to many EIs. However, other HIV-1 isolates under EI pressure may select for mutational pathways that confer specific drug resistance mechanisms that are unrelated to the general process of entry and may result in decrease fitness, as is the case with most drug resistance mutations.

B. Nucleoside/Nucleotide Reverse Transcriptase Inhibitors

1. Resistance

Nucleoside analogue reverse transcriptase inhibitors (NRTI) were the first class of drugs to be approved by the Food and Drug Administration of the United States (FDA) (e.g., zidovudine, AZT was approved in 1987) (Young, 1988).

NRTIs are administered as prodrugs, which require host cell entry and triphosphorylation (Furman *et al.*, 1986; Hart *et al.*, 1992; Mitsuya and Broder, 1986; Mitsuya *et al.*, 1985; St Clair *et al.*, 1987) by cellular kinases prior to enacting an antiviral effect. Lack of a 3′-hydroxyl group at the sugar (2′-deoxyribosyl) moiety of the NRTI prevents the formation of a 3′–5′ phosphodiester bond between the NRTI and incoming 5′-nucleoside triphosphates. Chain termination via incorporation of the NRTI-tripshosphate can occur during RNA-dependent DNA or DNA-dependent DNA synthesis catalyzed by RT to produce the (−) and (+) strands, respectively, of proviral DNA (Balzarini *et al.*, 1989; Cheng *et al.*, 1987; De Clercq, 2001; Richman, 2001). Phosphorylated NRTIs are also strong competitors of native deoxyribonucleotide triphosphates (dNTPs) for binding to HIV RT but this competition is affected by the dNTP pools and efficiency of nucleoside anabolic pathways (De Clercq, 2001).

To date, there are eight FDA approved NRTIs: abacavir (ABC, Ziagen), didanosine (ddI, Videx), emtricitabine (Emtriva), lamivudine (3TC, Epivir), stavudine (d4T, Zerit), zalcitabine (ddC, Hivid), zidovudine (AZT, Retrovir), and tenofovir disoprovil fumarate (TDF, Viread), a nucleotide RT inhibitor. Treatment with these drugs often results in the emergence of resistant HIV-1 strains with reduced drug susceptibility secondary to amino acid changes. These amino acid changes could be sequential additions (e.g., for AZT resistance), insertions, or single amino acid substitutions in RT (e.g., for 3TC resistance) (see Fig. 3).

HIV resistance to NRTI is mediated by two mechanisms: ATP-dependent pyrophosphorolysis, which is the removal of NRTIs from the 3′-end of the nascent chain and reversal of chain termination (Arion *et al.*, 1998; Boyer *et al.*, 2001; Meyer *et al.*, 1999) and increased discrimination between native deoxyribonucleotides and NRTI. Mutations that increase NRTI excision by pyrophosphorolysis are the thymidine analogue mutations (TAMs). These amino acid changes (especially D67N, K70R, and 215) promote pyrophosphorolysis and are involved in the removal of AZT and d4T from viral DNA chains (Arion *et al.*, 1998; Meyer *et al.*, 2002; Naeger *et al.*, 2002). Thus, NRTI mutations can be classified as nucleoside/nucleotide-associated mutations (NAMs) or TAMs. TAMs are a subset of NAMs (Johnson *et al.*, 2005). TAM amino acid changes in HIV-1 RT include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E (Bacheler *et al.*, 2001; Boucher *et al.*, 1992; Harrigan *et al.*, 1996; Kellam *et al.*, 1992; Larder and Kemp, 1989). It has been suggested that these mutations occur by two distinct pathways: TAM1 and TAM2. TAM1 pathway includes M41L, L210W, T215Y, and occasionally D67N while the TAM2 pathway includes D67N, K70R, T215F, and 219E/Q (Bacheler *et al.*, 2001; Marcelin *et al.*, 2004; Yahi *et al.*, 2005).

The second pathway associated with NRTI resistance is the prevention of NRTI incorporation into the nascent chain. Mutations associated with

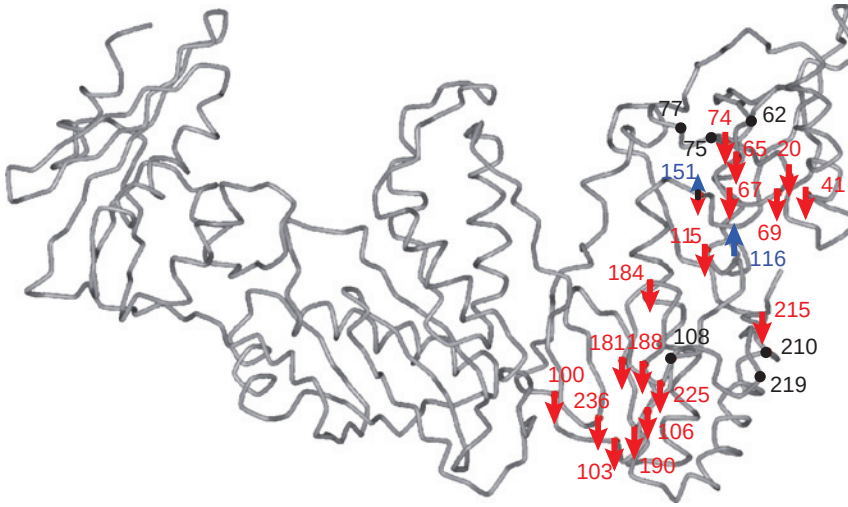


FIGURE 3 Location of RTI resistance mutations in the HIV-1 RT crystal structure. All of the known drug resistance mutations conferring resistance to NRTIs and NNRTIs are mapped onto this RT crystal structure (Rodgers *et al*., 1995). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness. A blue arrow signifies a drug resistance mutation associated with an increase in replicative fitness.

this mechanism include the M184V/I and the K65R. M184 amino acid is located in the highly conserved nucleotide binding pouch in HIV-1 RT, and thus a mutation at this point is associated with defective enzyme activity (see the later description). M184V mutation emerges with 3TC or FTC therapy and confers more than 100-fold increase in resistance to 3TC in particular and is associated with viral rebound (Quan *et al*., 1996; Schinazi *et al*., 1993). In the presence of M184V, two or three TAMs are required for the emergence of phenotypic resistance to zidovudine. M184V rarely occurs in the course of AZT treatment and usually signifies high-level zidovudine resistance. M184V restores tenofovir susceptibility in the presence of K65R (Deval *et al*., 2004). M184V associated with 67% of the activity of wild-type RT while M184I associated with 43% of wild-type RT (Back *et al*., 1996). This decrease in enzymatic activity mediated by specific mutations also affects the replicative fitness of the virus (in a common genetic backbone) (see the later description). Treatment with tenofovir can select for the K65R mutations, which confers a three- to four-fold decrease in susceptibility to tenofovir (Margot *et al*., 2002; Shehu-Xhilaga *et al*., 2005; Wainberg *et al*., 1999). The K65R mutation has also been identified with resistance to ddC, ddI, d4T, and ABC (Garcia-Lerma *et al*., 2003). In general, K65R rarely emerges in patients receiving any AZT-containing

regimen since this mutation is phenotypically antagonistic to the TAMs (Parikh *et al.*, 2006; White *et al.*, 2006).

Multidrug-resistant mutations (MDR) were first characterized with the following amino acid changes: Q151M, F116Y, F77L, V75I, and A62V (Iversen *et al.*, 1996; Shafer *et al.*, 1994; Shirasaka *et al.*, 1995), which usually emerge in patients treated with AZT in combination with either d4T, ddC, or ddI (Iversen *et al.*, 1996; Shirasaka *et al.*, 1995). The Q151M mutation is the first and pivotal mutation to appear in this series of mutations. Also associated with zidovudine treatment and TAMs is the MDR-69 complex, which involves a deletion of an amino acid at position 67 linked to the 69G/70R/74I/103N/215F/219Q genotype in RT (Imamichi *et al.*, 2001). Another position associated with mutations is the insertion of SS, SG, or SA between amino acids 69 and 70 in a background of TAMs associated with AZT and d4T resistance (De Antoni *et al.*, 1997; de Jong *et al.*, 1999; Larder *et al.*, 1999; Tamalet *et al.*, 1998). The 69 insertion complex has been identified following prolonged AZT treatment with ddI or ddC in ~2% of treated patients (Van Vaerenbergh *et al.*, 2000).

2. *Viral Fitness*

Figure 3 summarizes a list of RTI-resistant mutations and their effect on viral replicative fitness. A large number of primary and secondary mutations (or combinations of these) in the RT enzyme have been shown to increase fitness over the wild-type virus (Quinones-Mateu and Arts, 2002a,b, 2006). The potential for a clinical benefit associated with reduced replicative fitness of NRTI-resistant HIV-1 variants has not been addressed to the same degree as for the PI (see the later description). For example, reversion of most NRTI-resistant mutations is slower than most PI-resistant substitutions in the absence of therapy implying some compensation by secondary mutations.

It is not surprising that the first series of studies describing the effect of drug-resistance mutations on viral fitness were related to zidovudine (de *et al.*, 1996; Goudsmit *et al.*, 1996; Harrigan *et al.*, 1998; Imamichi *et al.*, 2000; Kosalaraksa *et al.*, 1999; Maeda *et al.*, 1998). In AZT-experienced patients, the wild-type 215T HIV-1 isolate eventually reemerged from the diverse quasispecies and outcompeted the AZT-resistant 215Y strain when AZT was discontinued (Goudsmit *et al.*, 1996). Long-term failure of AZT is associated with the stepwise accumulation of AZT resistance mutations (70R, 215Y, and 41L) (de *et al.*, 1996; Larder and Kemp, 1989). Moreover, the ordered accumulation of resistant variants observed *in vivo* was predicted by the changes in relative fitness observed *in vitro* (i.e., wt > 70R $\gg$ 215Y = 41L/215Y > 41L) (Clavel *et al.*, 2000; Harrigan *et al.*, 1998; Imamichi *et al.*, 2000; Kosalaraksa *et al.*, 1999). Each additional AZT-associated mutation appears to increase the level of drug resistance (i.e., 10-fold with 70R to >100-fold with 41L, 67N, 70R, 215Y, and 219L).

Improvements in replicative capacity per se have not been clearly shown with these mutations.

D67N and K70R are found near the dNTP-binding site of RT whereas T215Y, T215F, and K219E are positioned near the primer-binding site. Both TAM-1 and TAM-2 pathways involve mutations that would lead to reduced HIV-1 RT processivity and the resulting decrease in replicative fitness observed with most AZT-resistant viruses (Caliendo *et al*., 1996). However, introduction of L210W and T215F into TAM-1 or TAM-2 backbones increased relative viral fitness in the presence of AZT pressure (Hu *et al*., 2006). Similarly, L210W and T215Y mutations are associated with lower replicative fitness in the absence of AZT, which seem to increase in the presence of the drug (Harrigan *et al*., 1996, 1998).

Resistance to lamivudine (mainly due to the M184V mutation) is perhaps the most studied relationship between HIV-1 replicative fitness and NRTI resistance (Back *et al*., 1996; Deval *et al*., 2004; Devereux *et al*., 2001; Martinez-Picado *et al*., 1999). Failure of lamivudine monotherapy has been associated with persistent decreases in viremia even though the virus exhibits high-level drug resistance (Kavlick *et al*., 1995; Larder *et al*., 1995; Pluda *et al*., 1995; Wainberg *et al*., 1995). Several mechanisms have been proposed to explain this observation, including (1) the lamivudine-associated 184V mutation appears to increase RT fidelity, thereby reducing mutation rates and the capacity of the virus to evolve (Quan *et al*., 1996; Wainberg *et al*., 1996), and (2) the M184V mutation appears to result in diminished RT processivity (Back *et al*., 1996; Miller *et al*., 1998) and, as a consequence, reduced replication capacity (Deval *et al*., 2004; Feng and Anderson, 1999; Picchio *et al*., 2000).

The tenofovir-associated K65R mutation appears to reduce viral replicative capacity at least to the same degree that the lamivudine-associated M184V mutation reduces it (Deval *et al*., 2004; Margot *et al*., 2002; Miller *et al*., 1998; Weber *et al*., 2005). The mechanism for this altered replicative capacity, however, appears to be unique (Deval *et al*., 2004; Weber *et al*., 2005). The fact that M184V is more commonly observed during failure of lamivudine compared with the rate K65R emerges during failure of tenofovir (White *et al*., 2002) may be due to far greater decreases in drug susceptibility associated with M184V compared with K65R. Thus, the M184V-containing virus seems to be far more fit in the presence of 3TC than the K65R-containing virus is in the presence of tenofovir.

In general, the Q151 complex of mutations in multidrug-resistant viruses has been associated with increased viral replicative capacity (Garcia-Lerma *et al*., 2003; Kosalaraksa *et al*., 1999; Maeda *et al*., 1998; Schmit *et al*., 1996). However, specific combinations of these mutations may reduce viral replicative fitness in the absence of drug pressure (Garcia-Lerma *et al*., 2003; Iversen *et al*., 1996; Maeda *et al*., 1998; Schmit *et al*., 1996). For example, viruses containing the F77L and Q151M changes have been

associated with reduced replicative fitness that is partially restored by the emergence of V75I (Iversen *et al.*, 1996). Q151L and Q151K have been shown to be intermediate mutations with lower replicative fitness than the Q151M mutation found in MDR complex (Garcia-Lerma *et al.*, 2003).

The MDR-69 complex in NRTI-resistant viruses is associated with reduced replicative fitness. It has been suggested that the increased viral fitness associated with the 69 insertion in a background of TAMs is secondary to T215 change (Asn-215, Ser-215, or Thr-215) (Prado *et al.*, 2004). It has been shown, however, that the 69SS insertion exerts limited impact on viral fitness as the removal of this mutation has further reduced viral fitness in the absence of drugs (Quinones-Mateu *et al.*, 2002).

C. Non-Nucleoside Reverse Transcriptase Inhibitors

1. Resistance

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are compounds that inhibit HIV-1 RT by binding to it and forming a hydrophobic pocket proximal to, but not overlapping, the polymerase active site on the enzyme (Kohlstaedt *et al.*, 1992; Tantillo *et al.*, 1994). NNRTIs do not possess the nucleoside structures that require intracellular metabolism to activate products. Unlike NRTIs, these non/uncompetitive inhibitors bind specifically to HIV-1 RT and do not inhibit the RT of other lentiviruses such as HIV-2 and SIV (De Clercq, 2001; Kohlstaedt *et al.*, 1992; Witvrouw *et al.*, 1999). The binding site of NNRTIs is distal from the active site of RT (Smerdon *et al.*, 1994). NNRTIs interact with a nonsubstrate-binding site proximal to the active site catalytic residues (aspartic acid triad) of HIV-1 RT, thus changing the spatial conformation of the substrate-binding site and reducing the polymerase activity of HIV-1 reverse transcription (Kohlstaedt *et al.*, 1992; Spence *et al.*, 1995). Rapid emergence of high-level NNRTIs associated resistance usually results from single amino acid substitutions, located directly in the noncatalytic, NNRTI-binding pocket in HIV-1 RT (Tantillo *et al.*, 1994). This NNRTI-binding pocket exists only in the presence of NNRTIs and is not "open" in the unliganded enzyme (Hsiou *et al.*, 1996; Rodgers *et al.*, 1995). The binding pocket consists of hydrophobic residues (Y181, Y188, F227, W229, and Y232), and hydrophilic residues such as K101, K103, S105, D192, and E224 of the p66 subunit and E138 of the p51 subunit (Sluis-Cremer *et al.*, 2004). The entrance to the NNRTI-binding pocket is ringed by an interface at the p66/p51 junction (Hsiou *et al.*, 1996). Mutations in L100, K101, K103, E138, V179, Y181, and Y188 have been associated with NNRTI cross-resistance (Antinori *et al.*, 2002). However, the most common NNRTI mutations are K103N and Y181C (Bacheler *et al.*, 2000, 2001; Demeter *et al.*, 2000; Dykes *et al.*, 2001).

The resistant mutation, Y181C, has been known to persist through multiple passages in drug free medium (Richman *et al.*, 1991). Y181C mutation has also been associated with suppression of AZT resistance (Richman *et al.*, 1991). The Y181 amino acid residue lies along with other hydrophobic amino acids (Y188, F227, W229, and Y232) in the NNRTI-binding pocket (NNRTI-bp) which is located near, but not overlapping, the active site of HIV-1 RT (Hsiou *et al.*, 1996; Larder, 1992; Rodgers *et al.*, 1995). Y181C is associated with intermediate to high-level resistance to nevirapine (NVP) with cross-resistance to delavirdine (DLV) and has been identified in 5% of NNRTI-treated patients (Dueweke *et al.*, 1993; Richman *et al.*, 1991). Efavirenz (EFV), a second generation NNRTI, is smaller than NVP and DLV without accessing the Y181 site such that Y181C only confers low-level resistance to EFV. K103N mutation occurs in more than 50% of patients on EFV and 30% of patients on NVP therapy. G190S/A/E develops in 10% of patients receiving EFV and 15% of patients on NVP therapy.

As with NRTI resistance, rare and complex patterns of NNRTI-resistant mutations can arise and confer high level of resistance. The set of I135L, T139V, and V245T substitutions conferred >1000-fold resistance to NVP and DLV in a subtype D HIV-1 infected individual not receiving antiretroviral treatment (Gao *et al.*, 2004). I135L and T139V are thought to confer resistance through the noncatalytic p51 subunit. Although I135L has been identified in subtype B HIV-1 infected individuals (Brown *et al.*, 2000), it is clear that alternative NNRTI resistance pathways are observed in nonsubtype B infected individuals (Brenner *et al.*, 2003; Gao *et al.*, 2004; Spira *et al.*, 2003).

2. *Viral Fitness*

A single dose of NNRTI may be enough to select drug-resistant viruses, as a single nucleotide change usually results in high-level resistance to this class of drugs (Deeks, 2001). This high-level resistance generally has either no effect or results in a slight reduction in replicative fitness of drug-resistant strains (Dykes *et al.*, 2001; Imamichi *et al.*, 2001). Available data suggest that single-point mutations such as 103N or 181C, selected during NNRTI treatment, have limited effects on viral fitness but confer high-level resistance and persist in the absence of drug pressure. Unlike other ARVs, the genetic barrier to NNRTI resistance is thought to be minimal and may account for the very high frequency of NNRTI mutations in the infecting HIV-1 population during incomplete viral suppression with NNRTI-based regimens. Subdominant NNRTI resistance virus in a patient HIV-1 population is most evident in mother–infant pairs treated with NVP (Eshleman *et al.*, 2005; Flys *et al.*, 2005).

The lack of a fitness barrier to NNRTI resistance is not surprising when considering where and how these drugs work. The NNRTI-binding pocket is

distinct from the active site of RT, and has only a structural role in the catalytic activity of RT. Thus, changes that maintain the general hydrophobicity and architecture of the pocket will likely confer high-level resistance without a negative impact on enzyme function. NNRTI mutations have been associated with defective RNase H activity with slowing of 3′-DNA and 5′-RNA RNase H cleavage (Archer *et al.*, 2000; Gerondelis *et al.*, 1999), and this effect has been correlated to the reduced viral fitness (Archer *et al.*, 2000). In addition to impacting RNase H activity, the G190E substitution has been associated with reduced RT polymerase activity and processivity (Boyer *et al.*, 1998; Fan *et al.*, 1996).

Related lentiviral polymerases (e.g., HIV-1 group O and HIV-2) sharing conserved structural RT domains are naturally resistant to NNRTI. Most HIV-1 group O isolates are intrinsically resistant to NNRTI due to the presence of three amino acid substitutions (i.e., 98G, 179E, and 181C) in RT, which obviously do not affect the wild-type replicative fitness (Descamps *et al.*, 1997; Quinones-Mateu *et al.*, 1997). The low barrier for resistance and the lack of distinct function associated with the NNRTI-binding pocket may also result in divergent evolution and the emergence of HIV-1 isolates that are intrinsically resistant to NNRTIs. Most subtypes A and D HIV-1 isolates in Uganda appear to have "wild-type" susceptibility to NNRTIs but one subtype D HIV-1 outlier was insensitive to NVP and DLV inhibition and showed weak inhibition by EFV (Gao *et al.*, 2004). This subtype D HIV-1 isolate with three resistance-associated amino acids (135L, 139V, and 245T) had wild-type fitness even though the RT activity was reduced. These studies again suggest that reduction in activity of a specific enzymatic function or replication step may be easily compensated throughout the HIV-1 genome.

D. Integrase Inhibitors

1. Resistance

Two integrase inhibitors (INIs), Raltegravir, MK-0518 (Merck) and Elvitegravir, GS-9137 (Gilead) are currently in various stages of clinical trials. On the basis of clinical studies with Raltegravir, resistance to this strand transfer inhibitor was conferred by two different sets of mutations in the integrase gene (Cooper *et al.*, 2007; Steigbigel *et al.*, 2007). The resistance genotype involves the primary resistance mutation N155H along with several secondary mutations including E92Q, V151L, T97A, G163R, and L74M (see Fig. 4). A second pathway was related to primary mutations at position 148 (i.e., Q148K/R/H) and linked to secondary mutations such as G140S/A and E138K.

Early *in vitro* studies suggested that accumulation of resistant mutations during selection with diketo acid inhibitors in the integrase would reduce

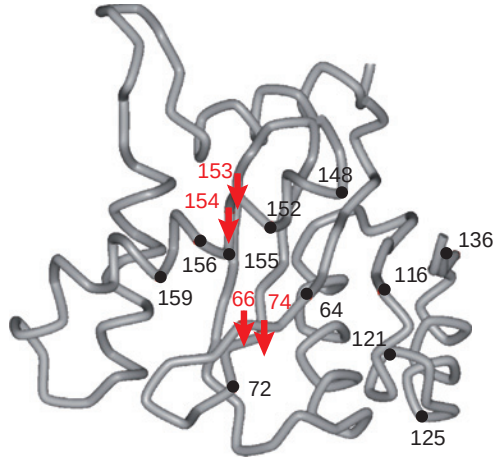


FIGURE 4 Location of INI resistance mutations in the IN core crystal structure. The mutations selected during *in vitro* passage with INIs or selected during treatment with MK-0518 are mapped onto the IN core crystal structure (Goldgur *et al.*, 1998). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.

viral replication (Fikkert *et al.*, 2003). *In vitro* passages with the INI L-708906 are selected for the following mutations: T66I + S153Y and T66I + M154I (Hazuda *et al.*, 2000) or T66I + S153Y + N155S (Hazuda *et al.*, 2004a). Fikkert *et al.* (2003) also identified mutations T66I, L74M, and S230R selected under L-708906 pressure. Another DKA, L-870812 selected N155H as the major drug resistance mutation in Rhesus macaques after a month of exposure to the compound (Hazuda *et al.*, 2004b). An azole derivative of DKA, S1360, selected the following amino acid substitutions: T66I, L74M, Q146K, Q148K, I151L, and N155S as majority mutations while the following changes, A128T, E138K, S153A, K160D, V165I, and V201I, were detected as a mixture with the wild-type amino acids (Fikkert *et al.*, 2004). Finally, L-870810, a naphthyridine carboxamide, selected mutations V72I, F121Y, T125K, and V151I sequentially over a period of 9 months (Hazuda *et al.*, 2004a). To date, the involvement of each of these substitutions in resistance to the various INIs has not been fully resolved.

2. *Viral Fitness*

The effects of resistance mutations to HIV-1 INIs on viral replicative fitness have yet to be fully analyzed. Initial *in vitro* selection of viruses resistant to the diketo acid L-708906 showed that triple mutant variants (i.e., T66I + L74M + S230R) have impaired 3′ processing and strand transfer activities (Fikkert *et al.*, 2003). This effect in enzymatic activity

was associated with a decrease in the replication kinetics when compared with the wild-type HIV-1 strain. Similar results were obtained during *in vitro* passage of HIV-1_{IIIB} in the presence of increasing concentrations of the diketo analogue S-1360 (Fikkert *et al.*, 2004). A total of nine amino acid substitutions were identified in the catalytic domain of the integrase, including T66I and L74M, which have been associated with resistance to L-708906. Reduced replication fitness was observed for all mutant strains compared with the wild-type strain (Fikkert *et al.*, 2004). Finally, the N155S mutation, which confers cross-resistance to both DKAs and naphthyridine carboxamide, has been associated with a 70% reduction on replicative capacity as measured in single-cycle assays (Hazuda *et al.*, 2004a). As is the case with the EI, maraviroc, the success of phase III clinical trials with Raltegravir (MK-0518) and likely approval by the FDA will likely lead to new studies on the effect of Raltegravir resistance on replicative fitness.

E. Protease Inhibitors

1. Resistance

The HIV-1 protease is the enzyme responsible for the cleavage of the viral Gag and Gag-Pol polyprotein precursors during virion maturation, which yields the structural proteins and the enzymes of the viral particle (Miller, 2001; Park and Morrow, 1993). Because of its vital role in the life cycle of HIV-1 and relatively small size (11 kDa), it was initially expected that resistance to PIs would be infrequent and uncommon during treatment. Ten PIs are currently approved by the FDA for use in the clinic: amprenavir (APV, Agenerase), atazanavir (ATZ, Reyataz), darunavir (TMC114, Prezista), fosamprenavir (Lexiva), indinavir (IDV, Crixivan), lopinavir (LPV), nelfinavir (NFV, Viracept), ritonavir (RTV, Norvir), saquinavir (SQV, Fortovase/Invirase), and tipranavir (TPV, Aptivus). The protease gene has shown great plasticity, with polymorphisms detected in 49 of the 99 amino acids of the HIV-1 protease monomer and more than 20 substitutions directly associated with resistance to PIs (Shafer *et al.*, 2000) (Fig. 5). Interestingly, primary drug-resistant substitutions rarely dominate the quasispecies in PI-naïve HIV-infected individuals (Kozal *et al.*, 1996), suggesting that they confer a selective disadvantage to the virus (see the later description). In fact, emergence of PI resistance seems to require the stepwise accumulation of primary and compensatory mutations (Molla *et al.*, 1996a), where each inhibitor usually selects for certain signature primary mutations and a characteristic pattern of compensatory mutations.

All PIs share relatively similar chemical structures and cross-reactivity is commonly observed in the clinical setting. Multiple mutations appear to be necessary for the development of resistance to PI while maintaining virus maturation and replication. For most PIs, primary PI resistance mutations

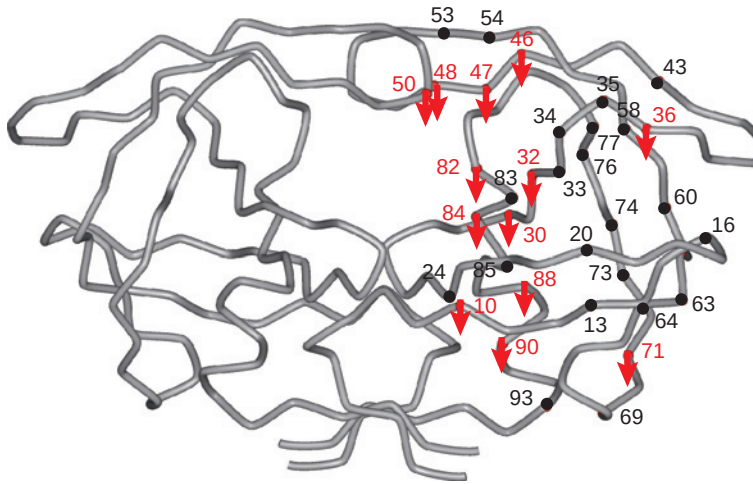


FIGURE 5 Location of PI resistance mutations in the protease crystal structure. The PI resistance mutations were mapped onto the PR core crystal structure (Wlodawer *et al.*, 1989). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.

cluster near the active site of the enzyme (Fig. 5) at positions located at the substrate/inhibitor-binding site (e.g., D30N, G48V, I50V, V82A, or I84V, among others). These amino acid changes usually have a deleterious effect on the replicative fitness of the resistant virus (Nijhuis *et al.*, 2001; Quinones-Mateu and Arts, 2002a,b, 2006). These effects can be rescued by secondary mutations (e.g., at positions 10, 32, 33, 46, 47, 63, 71, or 77), which partially compensate the impairment on HIV replication. These mutations are usually located outside of the substrate-binding region of the enzyme suggesting conformational adaptation to the primary changes in the active site (Fig. 5). (Eastman *et al.*, 1998; Ho *et al.*, 1994; Mammano *et al.*, 1998; Nijhuis *et al.*, 1998, 1999; Rose *et al.*, 1996).

In addition to mutations in the protease gene, changes located within eight major protease cleavage sites (i.e., *gag* and *pol* genes), have been associated with resistance to PI (Clavel *et al.*, 2000; Doyon *et al.*, 1996; Miller, 2001; Nijhuis *et al.*, 2001; Zhang *et al.*, 1997). Mutations in these regions provide better peptide substrates for the mutated protease, which partially compensate for the resistance-associated loss of viral fitness (Clavel *et al.*, 2000; Doyon *et al.*, 1996; Mammano *et al.*, 1998; Nijhuis *et al.*, 2001; Zennou *et al.*, 1998). Moreover, it has been reported that some PI-resistant viruses display defects in the processing of the RT enzyme (de la Carriere *et al.*, 1999), reducing the levels of RT in the virions and perhaps contributing to a reduction in viral fitness. Interestingly, AZT resistance mutations in the

RT can partially rescue the replicative defect of a PI-resistant virus, which could be relevant to the therapeutic control of HIV-1 infection (de la Carriere *et al.*, 1999).

The most recent PIs approved by the FDA are tipranavir and darunavir. *In vitro* passage of HIV-1 in the presence of tipranavir selected for viruses with the following mutations: L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, and I84V, as well as a mutation in the CA/SP1 *gag* cleavage site (Doyon *et al.*, 2005). In addition, tipranavir has been extensively studied in patients carrying multidrug-resistant viruses and failing multiple PI-based regimens. Phase II and III studies resulted in the identification of 21 amino acid substitutions at 16 different positions, some of them coinciding with the *in vitro* selected mutations (i.e., L10V, I13V, K20M/R/V, L33F, E35G, M36I, K43T, M46L, I47V, I54A/M/V, Q58E, H69K, T74P, V82L/T, N83D, and I84V) (Hicks *et al.*, 2006; Kohlbrenner and Hall, 2004). Interestingly, mutations D30N, I50V, and N88D seemed to be associated with hypersusceptibility to tipranavir (De Luca, 2007).

Darunavir, although structurally similar to amprenavir, binds particularly tight to the HIV protease, increasing the affinity of the drug for the enzyme (King *et al.*, 2004; Koh *et al.*, 2003). Contrary to other PIs, *in vitro* selection experiments with darunavir have been less successful. Viruses selected in the presence of 10 nM darunavir showed only a tenfold reduction in susceptibility to the inhibitor while carrying two mutations (i.e., R41T and K70E). Interestingly, site-directed mutants containing these amino acid substitutions were fully susceptible to darunavir (De Meyer *et al.*, 2005), suggesting that other genomic regions such as the *gag* cleavage sites may play a bigger role in resistance to this PI. Finally, after showing potent antiviral activity in phase II studies, phase III clinical trials were designed to test darunavir with ritonavir. *In vivo* mutations associated with resistance to darunavir/ritonavir included V11I, V32I, L33F, I47V, I50V, I54M/L, G73S, L76V, I84V, and L89V (DeMeyer *et al.*, 2007).

Finally, another PI brecanavir (GW640385), currently in clinical development, has shown good *in vitro* potency. However, passages in the presence of the drug selected for nine amino acid substitutions in the protease gene: L10F, G16E, E21K, A28S, M46I, F53L, Q58E, A71V, and V82I, and three substitutions in *gag* cleavage sites (i.e., L449F, R452K and P453T) (Yates *et al.*, 2006). In phase I studies, I84V was associated with resistance to brecanavir, particularly in the presence of I47V (Ford *et al.*, 2006).

In summary, after the introduction of PI-based antiretroviral treatment, the virus follows a "step-by-step" general pathway to overcome the drug selection: (1) acquisition of primary resistance mutations in the protease gene, (2) selection of secondary/compensatory protease mutations to repair the enzymatic function and rescue viral fitness, and (3) selection of mutations in the major cleavage sites of the Gag and Gag-Pol polyprotein precursors in order to restore protein processing and to increase production of

HIV-1 protease enzyme (Berkhout, 1999; Condra *et al*., 1995; Doyon *et al*., 1998; Molla *et al*., 1996b; Nijhuis *et al*., 2001). However, the exact evolutionary pathway will depend on the type of PI, the viral genetic background, and stochastic mutations.

2. *Viral Fitness*

Several comprehensive reviews have summarized the effect of PI resistance mutations on HIV-1 replicative fitness (Berkhout, 1999; Nijhuis *et al*., 2001; Quinones-Mateu and Arts, 2006). Thus, this review will highlight only a few fitness analyses on PI-resistant viruses, which have had a significant impact on our understanding of fitness and drug resistance.

Multiple studies have described a significant reduction in HIV-1 replicative fitness as a consequence of PI resistance. A list relating primary and secondary mutations and their effect of viral replicative fitness is provided in Fig. 5. Most of these mutations reduce the replicative fitness; although several amino acid substitutions seem to have no effect on the ability of the virus to replicate (Quinones-Mateu and Arts, 2002a,b, 2006). As described earlier, most primary PI resistant mutations cluster near the active site of the enzyme (Fig. 5), reducing both catalytic activity and viral replicative fitness. Secondary mutations within the protease gene compensate for the impairment on HIV replication by helping the enzyme to adapt to the primary changes in the active site (Barbour *et al*., 2002; Borman *et al*., 1996; Eastman *et al*., 1998; Mammano *et al*., 1998; Nijhuis *et al*., 1998). In addition, increases on PI resistance are often associated with substitutions in the protease cleavage sites (*gag* and *pol* genes), which provide better peptide substrates for the mutated protease and compensate for a potential loss in viral replicative fitness (Cote *et al*., 2001; Doyon *et al*., 1996; Watkins *et al*., 2003; Zennou *et al*., 1998). Although they appear sporadically in the viral quasispecies (Quinones-Mateu and Arts, 2002a,b, 2006), most primary mutations are rarely found in a PI-naïve HIV-infected individual, suggesting that they are associated with reduced replicative fitness (Shafer *et al*., 2000).

The effect of PI-resistant mutations on viral fitness can be inferred from the rate at which wild-type strains repopulate the quasispecies after antiretroviral therapy is withdrawn. A greater impairment of viral replication should result in faster wild-type repopulation once drug pressure is removed. Using this approach, Devereux *et al*. (2001) analyzed data from 11 patients enrolled in a study of treatment interruption during salvage therapy. Nelfinavir-selected D30N substitution and indinavir-selected M46I/L mutation were associated with a 12.4% and a 21% fitness reduction, respectively. Using a growth competition assay, Martinez-Picado *et al*. (1999) corroborated a substantial decrease in viral fitness in viruses harboring the nelfinavir-selected D30N mutation, relative to wild-type strains. Conversely, the L90M substitution only moderately decreased fitness. Taking a similar *in vivo* approach, Birk *et al*. (2001) reported that following withdrawal of

antiretroviral therapy, primary PI-resistant mutations were lost more rapidly than secondary PI mutations ($p < 0.05$) or primary RT mutations ($p < 0.01$), suggesting that primary PI resistance mutations impose the greatest impairment on viral replicative capacity.

Several studies have analyzed the effects of both ritonavir- and saquinavir-based therapy on viral replicative capacity and protease-mediated processing of Gag and Gag-Pol precursors. Zennou *et al.* (1998) found that all recombinant resistant mutant viruses harboring protease sequences from patients were less fit than recombinant clones carrying parental pretherapy proteases. This replicative defect involved mutations in the protease gene (46I, 48V, and 90M) and in at least one cleavage site (NC/p1). A similar study from the same group showed that mutations in the *gag* cleavage sites (MA/CA, CA/p2, and p1/p6) in patient-derived HIV-1 resistant variants only partially corrected for the loss of viral fitness due to the selection of RTV/SQV resistance mutations (Mammano *et al.*, 1998). Kaufmann *et al.* (2001) reported that insertions in the proline rich of the $p6^{gag}$ protein may affect the virologic response to RTV + SQV therapy. Thus, mutations in the *gag* cleavage sites (p7/p1 and p1/6) or C-terminal p6* residues serve as compensatory mutations to increase HIV-1 replicative capacity.

Few studies have analyzed the replication capacity of amprenavir-resistant viruses. Using both drug-resistant viruses selected *in vitro* and recombinant infectious clones; APV-resistant variants accumulated mutations at codons 10, 46, 47, 50, and 84 in the protease gene and in the *gag* p1/p6 cleavage site (codon 449) (Prado *et al.*, 2002). All the APV-mutant variants had an impaired replication capacity compared with the wild-type virus (wt > 10F > 10F/84V > 10F/46I/50V > 10F/46I/47V/50V) (Prado *et al.*, 2002). In contrast to most fitness studies on PI-resistant viruses where compensatory mutations increase viral replication capacity (Clavel *et al.*, 2000; Nijhuis *et al.*, 2001), in this study (Prado *et al.*, 2002), the progressive accumulation of PR mutations did not reestablish viral fitness.

Hellmann *et al.* (2002) characterized the clinical impact of HIV-1 fitness by analyzing data from 12 different studies, encompassing 800 clinical isolates from more than 500 patients. Viral replication capacity, relative to the $HIV\text{-}1_{NL4\text{-}3}$ wild-type reference, was assessed using the modified PhenoSense assay. Early HIV-1 infection typically involved wild-type virus with a broad range of replication capacities (i.e., <10–100%). Interestingly, recombinant viruses with lower replication capacity in early infection were associated with higher $CD4^+$ T-cell counts (Barbour *et al.*, 2002, 2004). Virologic failure was often accompanied by a sharp decline in replication capacity, particularly in patients with viruses resistant to PI, which correlated with the extent of viral suppression and increase in $CD4^+$ T-cell counts.

Reduction of HIV-1 fitness due to PI-resistant mutations has been cited as an explanation for the so-called "discordant response" to PI-containing therapy (i.e., preservation of $CD4^+$ T-cell counts despite virologic failure

and the presence of high viral loads). This phenomenon was studied in a prospective randomized pilot study in which patients with HIV RNA >2500 copies/ml were randomized to either continue ($n = 11$) or to discontinue therapy ($n = 5$) (Hellmann *et al.*, 2002). $CD4^+$ T-cell counts declined and viremia increased in patients who stopped therapy, accompanied by a switch to drug susceptibility and increase in viral fitness (as measured *in vivo*). In contrast, $CD4^+$ T-cell counts and viremia remained stable in patients who stayed on therapy, reflecting continued antiviral activity and maintenance of an unfit viral population. In a follow-up study, Stoddart and colleagues used PI-resistant HIV-1 isolates and PR-pseudotyped HIV-1 clones from clinical specimens to infect PBMC, human thymic organ cultures, and SCID-hu Thy/Liv mice (Stoddart *et al.*, 2001). Replication of PI-resistant strains was highly impaired in the thymus, suggesting a possible explanation for the preservation of $CD4^+$ T-cell counts in patients failing PI-based therapy. Collectively, these data clearly indicate that PI-associated mutations decrease replicative capacity, and that this decrease may have a significant impact on clinical outcomes. Ongoing *in vitro* and *in vivo* (clinical trials) studies with the most recently approved and investigational PIs (the effect of their mutations in viral replicative fitness is still under investigation) should aid in the design of novel antiretrovirals and provide rationale for appropriate treatment regimens.

References

Anastassopoulou, C. G., Marozsan, A. J., Matet, A., Snyder, A. D., Arts, E. J., Kuhmann, S. E., and Moore, J. P. (2007). Escape of human immunodeficiency virus type 1 from a small molecule CCR5 inhibitor is not associated with a fitness loss. *PLoS Pathog.* **3**(6), e79.

Antinori, A., Zaccarelli, M., Cingolani, A., Forbici, F., Rizzo, M. G., Trotta, M. P., Di Giambenedetto, S., Narciso, P., Ammassari, A., Girardi, E., De Luca, A., and Perno, C. F. (2002). Cross-resistance among nonnucleoside reverse transcriptase inhibitors limits recycling efavirenz after nevirapine failure. *AIDS Res. Hum. Retroviruses.* **18**, 835–838.

Archer, R. H., Dykes, C., Gerondelis, P., Lloyd, A., Fay, P., Reichman, R. C., Bambara, R. A., and Demeter, L. M. (2000). Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. *J. Virol.* **74**, 8390–8401.

Arien, K. K., Vanham, G., and Arts, E. J. (2007). Is HIV-1 evolving to a less virulent form in humans? *Nat. Rev. Microbiol.* **5**, 141–151.

Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998). Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): Increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* **37**, 15908–15917.

Baba, M., Miyake, H., Wang, X., Okamoto, M., and Takashima, K. (2007). Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. *Antimicrob. Agents Chemother.* **51**, 707–715.

Bacheler, L. T., Anton, E. D., Kudish, P., Baker, D., Bunville, J., Krakowski, K., Bolling, L., Aujay, M., Wang, X. V., Ellis, D., Becker, M. F., Lasut, A. L., *et al.* (2000). Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. *Antimicrob. Agents Chemother.* **44**, 2475–2484.

Bacheler, L., Jeffrey, S., Hanna, G., D'Aquila, R., Wallace, L., Logue, K., Cordova, B., Hertogs, K., Larder, B., Buckery, R., Baker, D., Gallagher, K., *et al.* (2001). Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. *J. Virol.* **75**, 4999–5008.

Back, N. K., Nijhuis, M., Keulen, W., Boucher, C. A., Oude Essink, B. O., van Kuilenburg, A. B., van Gennip, A. H., and Berkhout, B. (1996). Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J.* **15**, 4040–4049.

Ball, S. C., Abraha, A., Collins, K. R., Marozsan, A. J., Baird, H., Quinones-Mateu, M. E., Penn-Nicholson, A., Murray, M., Richard, N., Lobritz, M., Zimmerman, P. A., Kawamura, T., *et al.* (2003). Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C. *J. Virol.* **77**, 1021–1038.

Balzarini, J., Herdewijn, P., and De Clercq, E. (1989). Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J. Biol. Chem.* **264**, 6127–6133.

Barbour, J. D., Wrin, T., Grant, R. M., Martin, J. N., Segal, M. R., Petropoulos, C. J., and Deeks, S. G. (2002). Evolution of phenotypic drug susceptibility and viral replication capacity during long-term virologic failure of protease inhibitor therapy in human immunodeficiency virus-infected adults. *J. Virol.* **76**, 11104–11112.

Barbour, J. D., Hecht, F. M., Wrin, T., Segal, M. R., Ramstead, C. A., Liegler, T. J., Busch, M. P., Petropoulos, C. J., Hellmann, N. S., Kahn, J. O., and Grant, R. M. (2004). Higher CD4+ T cell counts associated with low viral pol replication capacity among treatment-naive adults in early HIV-1 infection. *J. Infect. Dis.* **190**, 251–256.

Beausejour, Y., and Tremblay, M. J. (2004). Susceptibility of HIV type 1 to the fusion inhibitor T-20 is reduced on insertion of host intercellular adhesion molecule 1 in the virus membrane. *J. Infect. Dis.* **190**, 894–902.

Berkhout, B. (1999). HIV-1 evolution under pressure of protease inhibitors: Climbing the stairs of viral fitness. *J. Biomed. Sci.* **6**, 298–305.

Birk, M., Svedhem, V., and Sonnerborg, A. (2001). Kinetics of HIV-1 RNA and resistance-associated mutations after cessation of antiretroviral combination therapy. *AIDS* **15**, 1359–1368.

Borman, A. M., Paulous, S., and Clavel, F. (1996). Resistance of human immunodeficiency virus type 1 to protease inhibitors: Selection of resistance mutations in the presence and absence of the drug. *J. Gen. Virol.* **77**, 419–426.

Boucher, C. A., O'Sullivan, E., Mulder, J. W., Ramautarsing, C., Kellam, P., Darby, G., Lange, J. M., Goudsmit, J., and Larder, B. A. (1992). Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. *J. Infect. Dis.* **165**, 105–110.

Boyer, P. L., Gao, H. Q., and Hughes, S. H. (1998). A mutation at position 190 of human immunodeficiency virus type 1 reverse transcriptase interacts with mutations at positions 74 and 75 via the template primer. *Antimicrob. Agents Chemother.* **42**, 447–452.

Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001). Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J. Virol.* **75**, 4832–4842.

Brenner, B., Turner, D., Oliveira, M., Moisi, D., Detorio, M., Carobene, M., Marlink, R. G., Schapiro, J., Roger, M., and Wainberg, M. A. (2003). A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS* **17**, F1–F5.

Brown, A. J., Precious, H. M., Whitcomb, J. M., Wong, J. K., Quigg, M., Huang, W., Daar, E. S., D'aquila, R. T., Keiser, P. H., Connick, E., Hellmann, N. S., Petropoulos, C. J., *et al.* (2000). Reduced susceptibility of human immunodeficiency virus type 1 (HIV-1) from patients with primary HIV infection to nonnucleoside reverse transcriptase inhibitors is associated with variation at novel amino acid sites. *J. Virol.* **74**, 10269–10273.

Caliendo, A. M., Savara, A., An, D., DeVore, K., Kaplan, J. C., and D'aquila, R. T. (1996). Effects of zidovudine-selected human immunodeficiency virus type 1 reverse transcriptase amino acid substitutions on processive DNA synthesis and viral replication. *J. Virol.* **70**, 2146–2153.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273.

Chang, T. L., Gordon, C. J., Roscic-Mrkic, B., Power, C., Proudfoot, A. E., Moore, J. P., and Trkola, A. (2002). Interaction of the CC-chemokine RANTES with glycosaminoglycans activates a p44/p42 mitogen-activated protein kinase-dependent signaling pathway and enhances human immunodeficiency virus type 1 infectivity. *J. Virol.* **76**, 2245–2254.

Cheng, Y. C., Dutschman, G. E., Bastow, K. F., Sarngadharan, M. G., and Ting, R. Y. (1987). Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. *J. Mol. Biol.* **262**, 2187–2189.

Clavel, F., Race, E., and Mammano, F. (2000). HIV drug resistance and viral fitness. *Adv. Pharmacol.* **49**, 41–66.

Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., and Shivaprakash, M. (1995). *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374**, 569–571.

Cooper, D., Gatell, J., Rockstroh, J., Katlama, C., Yeni, P., Lazzarin, A., Chen, J., Isaacs, R., Teppler, H., and Nguyen, B. (2007). Results of BENCHMRK-1, a Phase III study evaluating the efficacy and safety of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus. *In "14th Conference on Retroviruses and Opportunistic Infection* Program and Abstracts of the 14th Conference on Retroviruses and Opportunistic Infections; February 25–28, 2007; Los Angeles, CA." Oral 105aLB.

Cote, H. C., Brumme, Z. L., and Harrigan, P. R. (2001). Human immunodeficiency virus type 1 protease cleavage site mutations associated with protease inhibitor cross-resistance selected by indinavir, ritonavir, and/or saquinavir. *J. Virol.* **75**, 589–594.

De Antoni, A., Foli, A., Lisziewicz, J., and Lori, F. (1997). Mutations in the pol gene of human immunodeficiency virus type 1 in infected patients receiving didanosine and hydroxyurea combination therapy. *J. Infect. Dis.* **176**, 899–903.

De Clercq, E. (2001). "Antiretroviral Therapy." ASM Press, Washington, DC.

de Jong, J. J., Goudsmit, J., Lukashov, V. V., Hillebrand, M. E., Baan, E., Huismans, R., Danner, S. A., ten Veen, J. H., de Wolf, F., and Jurriaans, S. (1999). Insertion of two amino acids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs. *AIDS* **13**, 75–80.

de la Carriere, L. C., Paulous, S., Clavel, F., and Mammano, F. (1999). Effects of human immunodeficiency virus type 1 resistance to protease inhibitors on reverse transcriptase processing, activity, and drug sensitivity. *J. Virol.* **73**, 3455–3459.

De Luca, A. (2007). Resistance to newly approved and investigational protease inhibitors. *Curr. Opin. HIV AIDS* **2**, 130–136.

De Meyer, S., Azijn, H., Surleraux, D., Jochmans, D., Tahri, A., Pauwels, R., Wigerinck, P., and De Bethune, M. P. (2005). TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. *Antimicrob. Agents Chemother.* **49**, 2314–2321.

de, J., Veenstra, J., Stilianakis, N. I., Schuurman, R., Lange, J. M., de Boer, R. J., and Boucher, C. A. (1996). Host-parasite dynamics and outgrowth of virus containing a

single K70R amino acid change in reverse transcriptase are responsible for the loss of human immunodeficiency virus type 1 RNA load suppression by zidovudine. *Proc. Natl. Acad. Sci. USA* **93**, 5501–5506.

Deeks, S. G. (2001). International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. *J. Acquir. Immune. Defic. Syndr.* **26**, S25–S33.

Deeks, S. G., Lu, J., Hoh, R., Neilands, T. B., Beatty, G., Huang, W., Liegler, T., Hunt, P., Martin, J. N., and Kuritzkes, D. R. (2007). Interruption of enfuvirtide in HIV-1 infected adults with incomplete viral suppression on an enfuvirtide-based regimen. *J. Infect. Dis.* **195**, 387–391.

Demeter, L. M., Shafer, R. W., Meehan, P. M., Holden-Wiltse, J., Fischl, M. A., Freimuth, W. W., Para, M. F., and Reichman, R. C. (2000). Delavirdine susceptibilities and associated reverse transcriptase mutations in human immunodeficiency virus type 1 isolates from patients in a phase I/II trial of delavirdine monotherapy (ACTG 260). *Antimicrob. Agents Chemother.* **44**, 794–797.

DeMeyer, S., Vangeneugden, T., and Levebvre, E. (2007). Phenotypic and genotypic determinants of resistance to TMC-114: Pooled analysis of POWER 1, 2, 3. *Antivir. Ther.* **11**(Suppl 1), S83.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W. A., Ratner, L., Kappes, J. C., Shaw, G. M., and Hunter, E. (2000). Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* **74**, 8358–8367.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Zhang, Z., O'Brien, W. A., Ratner, L., Shaw, G. M., and Hunter, E. (2001). Sensitivity of human immunodeficiency virus type 1 to fusion inhibitors targeted to the gp41 first heptad repeat involves distinct regions of gp41 and is consistently modulated by gp120 interactions with the coreceptor. *J. Virol.* **75**, 8605–8614.

Descamps, D., Collin, G., Letourneur, F., Apetrei, C., Damond, F., Loussert-Ajaka, I., Simon, F., Saragosti, S., and Brun-Vezinet, F. (1997). Susceptibility of human immunodeficiency virus type 1 group O isolates to antiretroviral agents: *In vitro* phenotypic and genotypic analyses. *J. Virol.* **71**, 8893–8898.

Desmezieres, E., Gupta, N., Vassell, R., He, Y., Peden, K., Sirota, L., Yang, Z., Wingfield, P., and Weiss, C. D. (2005). Human immunodeficiency virus (HIV) gp41 escape mutants: Cross-resistance to peptide inhibitors of HIV fusion and altered receptor activation of gp120. *J. Virol.* **79**, 4774–4781.

Deval, J., White, K. L., Miller, M. D., Parkin, N. T., Courcambeck, J., Halfon, P., Selmi, B., Boretto, J., and Canard, B. (2004). Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J. Biol. Chem.* **279**, 509–516.

Devereux, H. L., Emery, V. C., Johnson, M. A., and Loveday, C. (2001). Replicative fitness *in vivo* of HIV-1 variants with multiple drug resistance-associated mutations. *J. Med. Virol.* **65**, 218–224.

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., and Lamarre, D. (1996). Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J. Virol.* **70**, 3763–3769.

Doyon, L., Payant, C., Brakier-Gingras, L., and Lamarre, D. (1998). Novel Gag-Pol frameshift site in human immunodeficiency virus type 1 variants resistant to protease inhibitors. *J. Virol.* **72**, 6146–6150.

Doyon, L., Tremblay, S., Bourgon, L., Wardrop, E., and Cordingley, M. G. (2005). Selection and characterization of HIV-1 showing reduced susceptibility to the non-peptidic protease inhibitor tipranavir. *Antiviral Res.* **68**, 27–35.

Dueweke, T. J., Pushkarskaya, T., Poppe, S. M., Swaney, S. M., Zhao, J. Q., Chen, I. S., Stevenson, M., and Tarpley, W. G. (1993). A mutation in reverse transcriptase of

bis(heteroaryl)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors. *Proc. Natl. Acad. Sci. USA* **90**, 4713–4717.

Dykes, C., Fox, K., Lloyd, A., Chiulli, M., Morse, E., and Demeter, L. M. (2001). Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants. *Virology* **285**, 193–203.

Eastman, P. S., Mittler, J., Kelso, R., Gee, C., Boyer, E., Kolberg, J., Urdea, M., Leonard, J. M., Norbeck, D. W., Mo, H., and Markowitz, M. (1998). Genotypic changes in human immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (Norvir) monotherapy. *J. Virol.* **72**, 5154–5164.

Eshleman, S. H., Guay, L. A., Mwatha, A., Brown, E., Musoke, P., Mmiro, F., and Jackson, J. B. (2005). Comparison of mother-to-child transmission rates in Ugandan women with subtype A versus D HIV-1 who received single-dose nevirapine prophylaxis: HIV Network for Prevention Trials 012. *J. Acquir. Immune. Defic. Syndr.* **39**, 593–597.

Fan, N., Rank, K. B., Slade, D. E., Poppe, S. M., Evans, D. B., Kopta, L. A., Olmsted, R. A., Thomas, R. C., Tarpley, W. G., and Sharma, S. K. (1996). A drug resistance mutation in the inhibitor binding pocket of human immunodeficiency virus type 1 reverse transcriptase impairs DNA synthesis and RNA degradation. *Biochemistry* **35**, 9737–9745.

Feng, J. Y., and Anderson, K. S. (1999). Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. *Biochemistry* **38**, 9440–9448.

Fikkert, V., Van Maele, B., Vercammen, J., Hantson, A., Van Remoortel, B., Michiels, M., Gurnari, C., Pannecouque, C., De Maeyer, M., Engelborghs, Y., De Clercq, E., Debyser, Z., *et al.* (2003). Development of resistance against diketo derivatives of human immunodeficiency virus type 1 by progressive accumulation of integrase mutations. *J. Virol.* **77**, 11459–11470.

Fikkert, V., Hombrouck, A., Van Remoortel, B., De Maeyer, M., Pannecouque, C., De Clercq, E., Debyser, Z., and Witvrouw, M. (2004). Multiple mutations in human immunodeficiency virus-1 integrase confer resistance to the clinical trial drug S-1360. *AIDS* **18**, 2019–2028.

Flys, T., Nissley, D. V., Claasen, C. W., Jones, D., Shi, C., Guay, L. A., Musoke, P., Mmiro, F., Strathern, J. N., Jackson, J. B., Eshleman, J. R., and Eshleman, S. H. (2005). Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. *J. Infect. Dis.* **192**, 24–29.

Ford, S. L., Reddy, Y. S., Anderson, M. T., Murray, S. C., Fernandez, P., Stein, D. S., and Johnson, M. A. (2006). Single-dose safety and pharmacokinetics of brecanavir, a novel human immunodeficiency virus protease inhibitor. *Antimicrob. Agents Chemother.* **50**, 2201–2206.

Furman, P. A., Fyfe, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S., and Mitsuya, H. (1986). Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**, 8333–8337.

Gao, Y., Paxinos, E., Galovich, J., Troyer, R., Baird, H., Abreha, M., Kityo, C., Mugyenyi, P., Petropoulos, C., and Arts, E. J. (2004). Characterization of a subtype D human immunodeficiency virus type 1 isolate that was obtained from an untreated individual and that is highly resistant to nonnucleoside reverse transcriptase inhibitors. *J. Virol.* **78**, 5390–5401.

Garcia-Lerma, J. G., MacInnes, H., Bennett, D., Reid, P., Nidtha, S., Weinstock, H., Kaplan, J. E., and Heneine, W. (2003). A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. *J. Virol.* **77**, 5685–5693.

Gerondelis, P., Archer, R. H., Palaniappan, C., Reichman, R. C., Fay, P. J., Bambara, R. A., and Demeter, L. M. (1999). The P236L delavirdine-resistant human immunodeficiency virus

type 1 mutant is replication defective and demonstrates alterations in both RNA 5′-end- and DNA 3′-end-directed RNase H activities. *J. Virol.* **73**, 5803–5813.

Goldgur, Y., Dyda, F., Hickman, A. B., Jenkins, T. M., Craigie, R., and Davies, D. R. (1998). Three new structures of the core domain of HIV-1 integrase: An active site that binds magnesium. *Proc. Natl. Acad. Sci. USA* **95**, 9150–9154.

Gordon, C. J., Muesing, M. A., Proudfoot, A. E., Power, C. A., Moore, J. P., and Trkola, A. (1999). Enhancement of human immunodeficiency virus type 1 infection by the CC-chemokine RANTES is independent of the mechanism of virus-cell fusion. *J. Virol.* **73**, 684–694.

Goudsmit, J., de Ronde, A., Ho, D. D., and Perelson, A. S. (1996). Human immunodeficiency virus fitness *in vivo*: Calculations based on a single zidovudine resistance mutation at codon 215 of reverse transcriptase. *J. Virol.* **70**, 5662–5664.

Guo, Q., Ho, H. T., Dicker, I., Fan, L., Zhou, N., Friborg, J., Wang, T., McAuliffe, B. V., Wang, H. G., Rose, R. E., Fang, H., Scarnati, H. T., *et al.* (2003). Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. *J. Virol.* **77**, 10528–10536.

Harrigan, P. R., Kinghorn, I., Bloor, S., Kemp, S. D., Najera, I., Kohli, A., and Larder, B. A. (1996). Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. *J. Virol.* **70**, 5930–5934.

Harrigan, P. R., Bloor, S., and Larder, B. A. (1998). Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. *J. Virol.* **72**, 3773–3778.

Hart, G. J., Orr, D. C., Penn, C. R., Figueiredo, H. T., Gray, N. M., Boehme, R. E., and Cameron, J. M. (1992). Effects of (−)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. *Antimicrob. Agents Chemother.* **36**, 1688–1694.

Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* **287**, 646–650.

Hazuda, D. J., Anthony, N. J., Gomez, R. P., Jolly, S. M., Wai, J. S., Zhuang, L., Fisher, T. E., Embrey, M., Guare, J. P., Jr., Egbertson, M. S., Vacca, J. P., Huff, J. R., *et al.* (2004a). A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proc. Natl. Acad. Sci. USA* **101**, 11233–11238.

Hazuda, D. J., Young, S. D., Guare, J. P., Anthony, N. J., Gomez, R. P., Wai, J. S., Vacca, J. P., Handt, L., Motzel, S. L., Klein, H. J., Dornadula, G., Danovich, R. M., *et al.* (2004b). Integrase inhibitors and cellular immunity suppress retroviral replication in rhesus macaques. *Science* **305**, 528–532.

Hellmann, N. S., Wrin, T., Bates, M., Grant, R. M., Hicks, C. B., Haubrich, R., and Petropoulos, C. J. (2002). Modelling the effect of HIV replication capacity on treatment outcomes. *Antivir. Ther.* **7**(Suppl 1), [Abstract no. 63 XI International HIV Drug Resistance Workshop, Seville, Spain] S53.

Hicks, C. B., Cahn, P., Cooper, D. A., Walmsley, S. L., Katlama, C., Clotet, B., Lazzarin, A., Johnson, M. A., Neubacher, D., Mayers, D., and Valdez, H. (2006). Durable efficacy of tipranavir-ritonavir in combination with an optimised background regimen of antiretroviral drugs for treatment-experienced HIV-1-infected patients at 48 weeks in the Randomized Evaluation of Strategic Intervention in multi-drug resistant patients with Tipranavir (RESIST) studies: An analysis of combined data from two randomised open-label trials. *Lancet* **368**, 466–475.

Ho, D. D., Toyoshima, T., Mo, H., Kempf, D. J., Norbeck, D., Chen, C. M., Wideburg, N. E., Burt, S. K., Erickson, J. W., and Singh, M. K. (1994). Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. *J. Virol.* **68**, 2016–2020.

Hsiou, Y., Ding, J., Das, K., Clark, A. D., Jr., Hughes, S. H., and Arnold, E. (1996). Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: Implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4**, 853–860.

Hu, Z., Giguel, F., Hatano, H., Reid, P., Lu, J., and Kuritzkes, D. R. (2006). Fitness comparison of thymidine analog resistance pathways in human immunodeficiency virus type 1. *J. Virol.* **80**, 7020–7027.

Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., Wilson, I. A., Wyatt, R., and Kwong, P. D. (2005). Structure of a V3-containing HIV-1 gp120 core. *Science* **310**, 1025–1028.

Imamichi, T., Berg, S. C., Imamichi, H., Lopez, J. C., Metcalf, J. A., Falloon, J., and Lane, H. C. (2000). Relative replication fitness of a high-level 3′-azido-3′-deoxythymidine-resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr → Gly) at codon 69. *J. Virol.* **74**, 10958–10964.

Imamichi, T., Murphy, M. A., Imamichi, H., and Lane, H. C. (2001). Amino acid deletion at codon 67 and Thr-to-Gly change at codon 69 of human immunodeficiency virus type 1 reverse transcriptase confer novel drug resistance profiles. *J. Virol.* **75**, 3988–3992.

Iversen, A. K., Shafer, R. W., Wehrly, K., Winters, M. A., Mullins, J. I., Chesebro, B., and Merigan, T. C. (1996). Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. *J. Virol.* **70**, 1086–1090.

Johnson, V. A., Brun-Vezinet, F., Clotet, B., Conway, B., Kuritzkes, D. R., Pillay, D., Schapiro, J., Telenti, A., and Richman, D. (2005). Update of the drug resistance mutations in HIV-1: 2005. *Top. HIV Med.* **13**, 51–57.

Kaufmann, G. R., Suzuki, K., Cunningham, P., Mukaide, M., Kondo, M., Imai, M., Zaunders, J., and Cooper, D. A. (2001). Impact of HIV type 1 protease, reverse transcriptase, cleavage site, and p6 mutations on the virological response to quadruple therapy with saquinavir, ritonavir, and two nucleoside analogs. *AIDS Res. Hum. Retroviruses* **17**, 487–497.

Kavlick, M. F., Shirasaka, T., Kojima, E., Pluda, J. M., Hui, F., Jr., Yarchoan, R., and Mitsuya, H. (1995). Genotypic and phenotypic characterization of HIV-1 isolated from patients receiving (−)-2′,3′-dideoxy-3′-thiacytidine. *Antiviral Res.* **28**, 133–146.

Kellam, P., Boucher, C. A., and Larder, B. A. (1992). Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proc. Natl. Acad. Sci. USA* **89**, 1934–1938.

King, N. M., Prabu-Jeyabalan, M., Nalivaika, E. A., Wigerinck, P., de Bethune, M. P., and Schiffer, C. A. (2004). Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. *J. Virol.* **78**, 12012–12021.

Kinomoto, M., Yokoyama, M., Sato, H., Kojima, A., Kurata, T., Ikuta, K., Sata, T., and Tokunaga, K. (2005). Amino acid 36 in the human immunodeficiency virus type 1 gp41 ectodomain controls fusogenic activity: Implications for the molecular mechanism of viral escape from a fusion inhibitor. *J. Virol.* **79**, 5996–6004.

Koh, Y., Nakata, H., Maeda, K., Ogata, H., Bilcer, G., Devasamudram, T., Kincaid, J. F., Boross, P., Wang, Y. F., Tie, Y., Volarath, P., Gaddis, L., *et al.* (2003). Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **47**, 3123–3129.

Kohlbrenner, V. M., and Hall, D. B. (2004). Tipranavir (TPV) mutation score predicts virologic success in protease inhibitors-experienced HIV-positive adults. HIV DART, Montego Bay, Jamaica, Abstract 040.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.

Kosalaraksa, P., Kavlick, M. F., Maroun, V., Le, R., and Mitsuya, H. (1999). Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an *in vitro* competitive HIV-1 replication assay. *J. Virol.* **73**, 5356–5363.

Kozal, M. J., Shah, N., Shen, N., Yang, R., Fucini, R., Merigan, T. C., Richman, D. D., Morris, D., Hubbel, E., Chee, M., and Gingeras, T. R. (1996). Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. *Nat. Med.* **2**, 753–759.

Kuhmann, S. E., Pugach, P., Kunstman, K. J., Taylor, J., Stanfield, R. L., Snyder, A., Strizki, J. M., Riley, J., Baroudy, B. M., Wilson, I. A., Korber, B. T., Wolinsky, S. M., *et al.* (2004). Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. *J. Virol.* **78**, 2790–2807.

Labrosse, B., Labernardiere, J., Dam, E., Trouplin, V., Skarabal, K., Clavel, F., and Mammano, F. (2003). Baseline susceptibility of primary human immunodeficiency virus type 1 to entry inhibitors. *J. Virol.* **77**, 1610–1613.

Labrosse, B., Morand-Joubert, L., Goubard, A., Rochas, S., Labernardiere, J. L., Pacanowski, J., Meynard, J. L., Hance, A. J., Clavel, F., and Mammano, F. (2006). Role of the envelope genetic context in the development of enfuvirtide resistance in human immunodeficiency virus type 1-infected patients. *J. Virol.* **80**, 8807–8819.

Larder, B. A. (1992). 3′-Azido-3′-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **36**, 2664–2669.

Larder, B. A., and Kemp, S. D. (1989). Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155–1158.

Larder, B. A., Kemp, S. D., and Harrigan, P. R. (1995). Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science* **269**, 696–699.

Larder, B. A., Bloor, S., Kemp, S. D., Hertogs, K., Desmet, R. L., Miller, V., Sturmer, M., Staszewski, S., Ren, J., Stammers, D. K., Stuart, D. I., and Pauwels, R. (1999). A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance. *Antimicrob. Agents Chemother.* **43**, 1961–1967.

Lin, P. F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y. F., Wang, H. G., Rose, R., Yamanaka, G., Robinson, B., Li, C. B., *et al.* (2003). A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proc. Natl. Acad. Sci. USA* **100**, 11013–11018.

Lobritz, M. A., Troyer, R. M., Marozsan, A. J., and Arts, E. J. (2007). Natural variation in the V3 crown of human immunodeficiency virus type 1 affects replicative fitness and entry inhibitor sensitivity. *J. Virol.* **81**, 8258–8269.

Lu, J., Sista, P., Giguel, F., Greenberg, M., and Kuritzkes, D. R. (2004). Relative replicative fitness of human immunodeficiency virus type 1 mutants resistant to enfuvirtide (T-20). *J. Virol.* **78**, 4628–4637.

Madani, N., Perdigoto, A. L., Srinivasan, K., Cox, J. M., Chruma, J. J., LaLonde, J., Head, M., Smith, A. B., III, and Sodroski, J. G. (2004). Localized changes in the gp120 envelope glycoprotein confer resistance to human immunodeficiency virus entry inhibitors BMS-806 and #155. *J. Virol.* **78**, 3742–3752.

Maeda, Y., Venzon, D. J., and Mitsuya, H. (1998). Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance. *J. Infect. Dis.* **177**, 1207–1213.

Mammano, F., Petit, C., and Clavel, F. (1998). Resistance-associated loss of viral fitness in human immunodeficiency virus type 1: Phenotypic analysis of protease and gag coevolution in protease inhibitor-treated patients. *J. Virol.* **72**, 7632–7637.

Marcelin, A. G., Delaugerre, C., Wirden, M., Viegas, P., Simon, A., Katlama, C., and Calvez, V. (2004). Thymidine analogue reverse transcriptase inhibitors resistance mutations profiles

and association to other nucleoside reverse transcriptase inhibitors resistance mutations observed in the context of virological failure. *J. Med. Virol.* **72**, 162–165.

Margot, N. A., Isaacson, E., McGowan, I., Cheng, A. K., Schooley, R. T., and Miller, M. D. (2002). Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF. *AIDS* **16**, 1227–1235.

Marozsan, A. J., Torre, V. S., Johnson, M., Ball, S. C., Cross, J. V., Templeton, D. J., Quinones-Mateu, M. E., Offord, R. E., and Arts, E. J. (2001). Mechanisms involved in stimulation of human immunodeficiency virus type 1 replication by aminooxypentane RANTES. *J. Virol.* **75**, 8624–8638.

Marozsan, A. J., Kuhmann, S. E., Morgan, T., Herrera, C., Rivera-Troche, E., Xu, S., Baroudy, B. M., Strizki, J., and Moore, J. P. (2005a). Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). *Virology* **338**, 182–199.

Marozsan, A. J., Moore, D. M., Lobritz, M. A., Fraundorf, E., Abraha, A., Reeves, J. D., and Arts, E. J. (2005b). Differences in the fitness of two diverse wild-type human immunodeficiency virus type 1 isolates are related to the efficiency of cell binding and entry. *J. Virol.* **79**, 7121–7134.

Martinez-Picado, J., Savara, A. V., Sutton, L., and D'Aquila, R. T. (1999). Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J. Virol.* **73**, 3744–3752.

Melby, T., Sista, P., DeMasi, R., Kirkland, T., Roberts, N., Salgo, M., Heilek-Snyder, G., Cammack, N., Matthews, T. J., and Greenberg, M. L. (2006). Characterization of envelope glycoprotein gp41 genotype and phenotypic susceptibility to enfuvirtide at baseline and on treatment in the phase III clinical trials TORO-1 and TORO-2. *AIDS Res. Hum. Retroviruses* **22**, 375–385.

Menzo, S., Castagna, A., Monachetti, A., Hasson, H., Danise, A., Carini, E., Bagnarelli, P., Lazzarin, A., and Clementi, M. (2004). Resistance and replicative capacity of HIV-1 strains selected *in vivo* by long-term enfuvirtide treatment. *New Microbiol.* **27**, 51–61.

Meyer, P. R., Matsuura, S. E., Mian, A. M., So, A. G., and Scott, W. A. (1999). A mechanism of AZT resistance: An increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol. Cell. Biol.* **4**, 35–43.

Meyer, P. R., Matsuura, S. E., Tolun, A. A., Pfeifer, I., So, A. G., Mellors, J. W., and Scott, W. A. (2002). Effects of specific zidovudine resistance mutations and substrate structure on nucleotide-dependent primer unblocking by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **46**, 1540–1545.

Miller, V. (2001). International perspectives on antiretroviral resistance. Resistance to protease inhibitors. *J. Acquir. Immune. Defic. Syndr.* **26**(Suppl 1), S34–S50.

Miller, M. D., Lamy, P. D., Fuller, M. D., Mulato, A. S., Margot, N. A., Cihlar, T., and Cherrington, J. M. (1998). Human immunodeficiency virus type 1 reverse transcriptase expressing the K70E mutation exhibits a decrease in specific activity and processivity. *Mol. Pharmacol.* **54**, 291–297.

Mitsuya, H., and Broder, S. (1986). Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**, 1911–1915.

Mitsuya, H., Weinhold, K. J., Furman, P. A., St. Clair, M. H., Lehrman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985). 3′-Azido-3′-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**, 7096–7100.

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., *et al.* (1996a).

Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat. Med.* **2**, 760–766.

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., *et al.* (1996b). Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat. Med.* **2**, 760–766.

Naeger, L. K., Margot, N. A., and Miller, M. D. (2002). ATP-dependent removal of nucleoside reverse transcriptase inhibitors by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **46**, 2179–2184.

Nameki, D., Kodama, E., Ikeuchi, M., Mabuchi, N., Otaka, A., Tamamura, H., Ohno, M., Fujii, N., and Matsuoka, M. (2005). Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. *J. Virol.* **79**, 764–770.

Neumann, T., Hagmann, I., Lohrengel, S., Heil, M. L., Derdeyn, C. A., Krausslich, H. G., and Dittmar, M. T. (2005). T20-insensitive HIV-1 from naive patients exhibits high viral fitness in a novel dual-color competition assay on primary cells. *Virology* **333**, 251–262.

Nijhuis, M., Boucher, C. A., Schipper, P., Leitner, T., Schuurman, R., and Albert, J. (1998). Stochastic processes strongly influence HIV-1 evolution during suboptimal protease-inhibitor therapy. *Proc. Natl. Acad. Sci. USA* **95**, 14441–14446.

Nijhuis, M., Schuurman, R., de Jong, D., Erickson, J., Gustchina, E., Albert, J., Schipper, P., Gulnik, S., and Boucher, C. A. (1999). Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* **13**, 2349–2359.

Nijhuis, M., Deeks, S., and Boucher, C. (2001). Implications of antiretroviral resistance on viral fitness. *Curr. Opin. Infect. Dis.* **14**, 23–28.

Parikh, U. M., Bacheler, L., Koontz, D., and Mellors, J. W. (2006). The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. *J. Virol.* **80**, 4971–4977.

Park, J., and Morrow, C. D. (1993). Mutations in the protease gene of human immunodeficiency virus type 1 affect release and stability of virus particles. *Virology* **194**, 843–850.

Picchio, G. R., Valdez, H., Sabbe, R., Landay, A. L., Kuritzkes, D. R., Lederman, M. M., and Mosier, D. E. (2000). Altered viral fitness of HIV-1 following failure of protease inhibitor-based therapy. *J. Acquir. Immune. Defic. Syndr.* **25**, 289–295.

Pluda, J. M., Cooley, T. P., Montaner, J. S., Shay, L. E., Reinhalter, N. E., Warthan, S. N., Ruedy, J., Hirst, H. M., Vicary, C. A., and Quinn, J. B. (1995). A phase I/II study of 2′-deoxy-3′-thiacytidine (lamivudine) in patients with advanced human immunodeficiency virus infection. *J. Infect. Dis.* **171**, 1438–1447.

Prado, J. G., Wrin, T., Beauchaine, J., Ruiz, L., Petropoulos, C. J., Frost, S. D., Clotet, B., D'aquila, R. T., and Martinez-Picado, J. (2002). Amprenavir-resistant HIV-1 exhibits lopinavir cross-resistance and reduced replication capacity. *AIDS* **16**, 1009–1017.

Prado, J. G., Franco, S., Matamoros, T., Ruiz, L., Clotet, B., Menendez-Arias, L., Martinez, M. A., and Martinez-Picado, J. (2004). Relative replication fitness of multi-nucleoside analogue-resistant HIV-1 strains bearing a dipeptide insertion in the fingers subdomain of the reverse transcriptase and mutations at codons 67 and 215. *Virology* **326**, 103–112.

Pugach, P., Marozsan, A. J., Ketas, T. J., Landes, E. L., Moore, J. P., and Kuhmann, S. E. (2007). HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. *Virology.* **361**, 212–228.

Quan, Y., Gu, Z., Li, X., Li, Z., Morrow, C. D., and Wainberg, M. A. (1996). Endogenous reverse transcription assays reveal high-level resistance to the triphosphate of (−)2′-dideoxy-3′-thiacytidine by mutated M184V human immunodeficiency virus type 1. *J. Virol.* **70**, 5642–5645.

Quinones-Mateu, M. E., and Arts, E. J. (2002a). Fitness of drug resistant HIV-1: Methodology and clinical implications. *Drug Resist. Updat.* **5**, 224–233.

Quinones-Mateu, M. E., and Arts, E. J. (2002b). HIV-1 fitness: Implications for drug resistance, disease progression, and global epidemic evolution. *In* "HIV Sequence Compendium 2001" (C. Kuiken, B. Foley, B. Hahn, P. Marx, F. McCutchan, J. Mellors, S. Wolinsky, and B. Korber, Eds.), pp. 134–170. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

Quinones-Mateu, M. E., and Arts, E. J. (2006). Virus fitness: Concept, quantification, and application to HIV population dynamics. *Curr. Top. Microbiol. Immunol.* **299**, 83–140.

Quinones-Mateu, M. E., Soriano, V., Domingo, E., and Menendez-Arias, L. (1997). Characterization of the reverse transcriptase of a human immunodeficiency virus type 1 group O isolate. *Virology* **236**, 364–373.

Quinones-Mateu, M. E., Ball, S. C., Marozsan, A. J., Torre, V. S., Albright, J. L., Vanham, G., van Der, G. G., Colebunders, R. L., and Arts, E. J. (2000). A dual infection/competition assay shows a correlation between *ex vivo* human immunodeficiency virus type 1 fitness and disease progression. *J. Virol.* **74**, 9222–9233.

Quinones-Mateu, M. E., Tadele, M., Parera, M., Mas, A., Weber, J., Rangel, H. R., Chakraborty, B., Clotet, B., Domingo, E., Menendez-Arias, L., and Martinez, M. A. (2002). Insertions in the reverse transcriptase increase both drug resistance and viral fitness in a human immunodeficiency virus type 1 isolate harboring the multi-nucleoside reverse transcriptase inhibitor resistance 69 insertion complex mutation. *J. Virol.* **76**, 10546–10552.

Rangel, H. R., Weber, J., Chakraborty, B., Gutierrez, A., Marotta, M. L., Mirza, M., Kiser, P., Martinez, M. A., Este, J. A., and Quinones-Mateu, M. E. (2003). Role of the human immunodeficiency virus type 1 envelope gene in viral fitness. *J. Virol.* **77**, 9069–9073.

Reeves, J. D., Gallo, S. A., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E., and Doms, R. W. (2003). Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc. Natl. Acad. Sci.* **99**, 16249–16254.

Reeves, J. D., Lee, F. H., Miamidian, J. L., Jabara, C. B., Juntilla, M. M., and Doms, R. W. (2005). Enfuvirtide resistance mutations: Impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization. *J. Virol.* **79**, 4991–4999.

Richman, D. D. (2001). HIV chemotherapy. *Nature* **410**, 995–1001.

Richman, D., Shih, C. K., Lowy, I., Rose, J., Prodanovich, P., Goff, S., and Griffin, J. (1991). Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88**, 11241–11245.

Rimsky, L. T., Shugars, D. C., and Matthews, T. J. (1998). Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. *J. Virol.* **72**, 986–993.

Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C., and Harrison, S. C. (1995). The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **92**, 1222–1226.

Rose, R. E., Gong, Y. F., Greytok, J. A., Bechtold, C. M., Terry, B. J., Robinson, B. S., Alam, M., Colonno, R. J., and Lin, P. F. (1996). Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors. *Proc. Natl. Acad. Sci. USA* **93**, 1648–1653.

Schinazi, R. F., Lloyd, R. M., Jr., Nguyen, M. H., Cannon, D. L., McMillan, A., Ilksoy, N., Chu, C. K., Liotta, D. C., Bazmi, H. Z., and Mellors, J. W. (1993). Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob. Agents Chemother.* **37**, 875–881.

Schmit, J. C., Cogniaux, J., Hermans, P., Van Vaeck, C., Sprecher, S., Van Remoortel, B., Witvrouw, M., Balzarini, J., Desmyter, J., De Clercq, E., and Vandamme, A. M. (1996). Multiple drug resistance to nucleoside analogues and nonnucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. *J. Infect. Dis.* **174**, 962–968.

Shafer, R. W., Kozal, M. J., Winters, M. A., Iversen, A. K., Katzenstein, D. A., Ragni, M. V., Meyer, W. A., III, Gupta, P., Rasheed, S., and Coombs, R. (1994). Combination therapy with zidovudine and didanosine selects for drug-resistant human immunodeficiency virus type 1 strains with unique patterns of pol gene mutations. *J. Infect. Dis.* **169**, 722–729.

Shafer, R. W., Dupnik, K., Winters, M. A., and Eshleman, S. H. (2000). A guide to HIV-1 reverse transcriptase and protease sequencing for drug resistance studies. *In* "HIV Sequence Compendium 2000" (C. Kuiken, B. Foley, B. Hahn, P. Marx, F. McCutchen, J. Mellors, S. Wolinsky, and B. Korber, Eds.). Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

Shehu-Xhilaga, M., Tachedjian, G., Crowe, S. M., and Kedzierska, K. (2005). Antiretroviral compounds: Mechanisms underlying failure of HAART to eradicate HIV-1. *Curr. Med. Chem.* **12**, 1705–1719.

Shirasaka, T., Kavlick, M. F., Ueno, T., Gao, W. Y., Kojima, E., Alcaide, M. L., Chokekijchai, S., Roy, B. M., Arnold, E., and Yarchoan, R. (1995). Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **92**, 2398–2402.

Sluis-Cremer, N., Temiz, N. A., and Bahar, I. (2004). Conformational changes in HIV-1 reverse transcriptase induced by nonnucleoside reverse transcriptase inhibitor binding. *Curr. HIV Res.* **2**, 323–332.

Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Chirino, A. J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1994). Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **91**, 3911–3915.

Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995). Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**, 988–993.

Spira, S., Wainberg, M. A., Loemba, H., Turner, D., and Brenner, B. G. (2003). Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance. *J. Antimicrob. Chemother.* **51**, 229–240.

St Clair, M. H., Richards, C. A., Spector, T., Weinhold, K. J., Miller, W. H., Langlois, A. J., and Furman, P. A. (1987). 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob. Agents Chemother.* **31**, 1972–1977.

Steigbigel, R., Kumar, P., Eron, J., Schechter, M., Markowitz, M., Loufty, M., Zhao, J., Isaacs, R., Nguyen, B., and Teppler, H. (2007). Results of BENCHMRK-2, a Phase III study evaluating the efficacy and safety of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus. *In* "*14th Conference on Retroviruses and Opportunistic Infection* Program and Abstracts of the 14th Conference on Retroviruses and Opportunistic Infections; February 25–28, 2007; Los Angeles, CA." Oral 105bLB.

Stoddart, C. A., Liegler, T. J., Mammano, F., Linquist-Stepps, V. D., Hayden, M. S., Deeks, S. G., Grant, R. M., Clavel, F., and McCune, J. M. (2001). Impaired replication of protease inhibitor-resistant HIV-1 in human thymus. *Nat. Med.* **7**, 712–718.

Tamalet, C., Izopet, J., Koch, N., Fantini, J., and Yahi, N. (1998). Stable rearrangements of the beta3-beta4 hairpin loop of HIV-1 reverse transcriptase in plasma viruses from patients receiving combination therapy. *AIDS* **12**, F161–F166.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A., and Arnold, E. (1994). Locations of anti-AIDS drug binding sites

and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *J. Mol. Biol.* **243**, 369–387.

Torre, V. S., Marozsan, A. J., Albright, J. L., Collins, K. R., Hartley, O., Offord, R. E., Quinones-Mateu, M. E., and Arts, E. J. (2000). Variable sensitivity of CCR5-tropic human immunodeficiency virus type 1 isolates to inhibition by RANTES analogs. *J. Virol.* **74**, 4868–4876.

Trkola, A., Gordon, C., Matthews, J., Maxwell, E., Ketas, T., Czaplewski, L., Proudfoot, A. E., and Moore, J. P. (1999). The CC-chemokine RANTES increases the attachment of human immunodeficiency virus type 1 to target cells via glycosaminoglycans and also activates a signal transduction pathway that enhances viral infectivity. *J. Virol.* **73**, 6370–6379.

Trkola, A., Kuhmann, S. E., Strizki, J. M., Maxwell, E., Ketas, T., Morgan, T., Pugach, P., Xu, S., Wojcik, L., Tagat, J., Palani, A., Shapiro, S., *et al.* (2002). HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. *Proc. Natl. Acad. Sci. USA* **99**, 395–400.

Van Vaerenbergh, K., Van Laethem, K., Albert, J., Boucher, C. A., Clotet, B., Floridia, M., Gerstoft, J., Hejdeman, B., Nielsen, C., Pannecouque, C., Perrin, L., Pirillo, M. F., *et al.* (2000). Prevalence and characteristics of multinucleoside-resistant human immunodeficiency virus type 1 among European patients receiving combinations of nucleoside analogues. *Antimicrob. Agents Chemother.* **44**, 2109–2117.

Wainberg, M. A., Salomon, H., Gu, Z., Montaner, J. S., Cooley, T. P., McCaffrey, R., Ruedy, J., Hirst, H. M., Cammack, N., and Cameron, J. (1995). Development of HIV-1 resistance to (−)2′-deoxy-3′-thiacytidine in patients with AIDS or advanced AIDS-related complex. *AIDS* **9**, 351–357.

Wainberg, M. A., Drosopoulos, W. C., Salomon, H., Hsu, M., Borkow, G., Parniak, M. A., Gu, Z., Song, Q., Manne, J., Islam, S., Castriota, G., and Prasad, V. R. (1996). Enhanced fidelity of 3TC.selected mutant HIV-1 reverse transcriptase. *Science* **271**, 1282–1285.

Wainberg, M. A., Miller, M. D., Quan, Y., Salomon, H., Mulato, A. S., Lamy, P. D., Margot, N. A., Anton, K. E., and Cherrington, J. M. (1999). *In vitro* selection and characterization of HIV-1 with reduced susceptibility to PMPA. *Antivir. Ther.* **4**, 87–94.

Watkins, T., Resch, W., Irlbeck, D., and Swanstrom, R. (2003). Selection of high-level resistance to human immunodeficiency virus type 1 protease inhibitors. *Antimicrob. Agents Chemother.* **47**, 759–769.

Weber, J., Chakraborty, B., Weberova, J., Miller, M. D., and Quinones-Mateu, M. E. (2005). Diminished replicative fitness of primary human immunodeficiency virus type 1 isolates harboring the K65R mutation. *Clin. Microbiol.* **43**, 1395–1400.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M., and Kappes, J. C. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* **46**, 1896–1905.

Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G., and Perros, M. (2007). Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J. Virol.* **81**, 2359–2371.

White, K. L., Margot, N. A., Wrin, T., Petropoulos, C. J., Miller, M. D., and Naeger, L. K. (2002). Molecular mechanisms of resistance to human immunodeficiency virus type 1 with reverse transcriptase mutations K65R and K65R + M184V and their effects on enzyme function and viral replication capacity. *Antimicrob. Agents Chemother.* **46**, 3437–3446.

White, K. L., Chen, J. M., Feng, J. Y., Margot, N. A., Ly, J. K., Ray, A. S., Macarthur, H. L., McDermott, M. J., Swaminathan, S., and Miller, M. D. (2006). The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. *Antivir. Ther.* **11**, 155–163.

Witvrouw, M., Pannecouque, C., Van Laethem, K., Desmyter, J., De Clercq, E., and Vandamme, A. M. (1999). Activity of non-nucleoside reverse transcriptase inhibitors against HIV-2 and SIV. *AIDS* **13**, 1477–1483.

Witvrouw, M., Fikkert, V., Hantson, A., Pannecouque, C., O'keefe, B. R., McMahon, J., Stamatatos, L., De, C. E., and Bolmstedt, A. (2005). Resistance of human immunodeficiency virus type 1 to the high-mannose binding agents cyanovirin N and concanavalin A. *J. Virol.* **79**, 7777–7784.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., and Kent, S. B. (1989). Conserved folding in retroviral proteases: Crystal structure of a synthetic HIV-1 protease. *Science* **245**, 616–621.

Yahi, N., Fantini, J., Henry, M., Tourres, C., and Tamalet, C. (2005). Structural analysis of reverse transcriptase mutations at codon 215 explains the predominance of T215Y over T215F in HIV-1 variants selected under antiretroviral therapy. *J. Biomed. Sci.* **12**, 701–710.

Yates, P. J., Hazen, R., St, C. M., Boone, L., Tisdale, M., and Elston, R. C. (2006). *In vitro* development of resistance to human immunodeficiency virus protease inhibitor GW640385. *Antimicrob. Agents Chemother.* **50**, 1092–1095.

Young, F. E. (1988). The role of the FDA in the effort against AIDS. *Public Health Rep.* **103**, 242–245.

Yusa, K., Maeda, Y., Fujioka, A., Monde, K., and Harada, S. (2005). Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. *J. Biol. Chem.* **280**, 30083–30090.

Zennou, V., Mammano, F., Paulous, S., Mathez, D., and Clavel, F. (1998). Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors *in vivo*. *J. Virol.* **72**, 3300–3306.

Zhang, Y. M., Imamichi, H., Imamichi, T., Lane, H. C., Falloon, J., Vasudevachari, M. B., and Salzman, N. P. (1997). Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. *J. Virol.* **71**, 6662–6670.

Mauro Giacca

Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano 99, 34012 Trieste, Italy

Gene Therapy to Induce Cellular Resistance to HIV-1 Infection: Lessons from Clinical Trials

I. Chapter Overview

Since the mid-1990s, a variety of approaches have been described having the capacity to suppress *human immunodeficiency virus-1* (HIV-1) infection or replication in cultured cells. These have included the expression of intracellular antibodies to viral proteins; of antisense RNAs that inhibit reverse transcription, processing, and translation of HIV-1 RNAs; of mutant HIV-1 structural or regulatory genes with dominant repressor activity; of RNA decoys that inhibit HIV-1 transcription; and of ribozymes targeting various HIV-1 RNA

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56010-2

species. More recently, the discovery of RNA interference (RNAi) and the identification of various cellular proteins that govern HIV-1 infection have offered additional technologies and novel targets for intervention, respectively. Taken together, the work performed in cell culture systems has indicated overall that the goal of rendering cells resistant to HIV-1 infection by gene transfer is attainable. A major challenge, however, has been the adaptation of these genetic strategies to a clinical setting. This chapter specifically focuses on the attempts made so far to render T cells resistant to HIV-1 infection and, more precisely, on the experience gained from the results of clinical trials. The purpose is to discuss the limits of the experimental approaches so far implemented, to describe the current status of research, and to critically highlight the advancements that are required before gene therapy of HIV-1 infection might eventually become clinically successful. Indeed, clinical success appears highly desirable today, especially in light of the limitations of current antiretroviral therapy in terms of long-term toxicity, emergence of drug-resistance HIV variants, and, most notably, inability to eradicate infection.

II. Introduction: Do We Need HIV-1 Gene Therapy?

The beginning of the gene therapy era (late 1980s) coincided with a period in which the molecular mechanisms that control HIV-1 replication were sufficiently deciphered to allow the design of gene-based strategies that might suppress viral infection. In those years, patients and clinicians were witnessing the poor performance of anti-HIV monotherapy, and the need for alternative approaches to treat HIV disease was urgent. During the first half of the 1990s, a dozen gene therapy clinical trials were initiated, aimed at achieving resolution of HIV infection by a variety of approaches (Bridges and Sarver, 1995). These included the induction of resistance in peripheral blood T cells or in hematopoietic progenitors, the *ex vivo* expansion of cytotoxic T cells recognizing HIV-infected lymphocytes, and the active immunization of patients against HIV antigens. The overall outcome of these trials has been largely disappointing. In particular, their results highlighted the paramount difference existing between the relative ease of inducing resistance to HIV infection in cell culture and the difficulty of achieving such a goal in infected patients.

The subsequent advent of the highly active antiretroviral therapy (HAART) era did then revolutionize the natural history of HIV infection and partially obscured the need for alternative therapies. Indeed, HAART has proven markedly successful in restoring or maintaining immune function and in reducing the risk of opportunistic disease and mortality (Palella *et al.*, 1998). In addition, the drugs available for HAART have progressively improved in recent years, allowing a significant reduction in the burden of pill taking and the emergence of undesired side effects (Dore and Cooper, 2006). At the end of the first decade since its introduction, however, the

initial enthusiasm for HAART is now giving way to several concerns of note. First, HAART is still fraught with important long-term toxicity, including an increased cardiovascular risk. Second, according to a recent survey involving the analysis of 12 European and North American prospective cohort studies, improvements in virologic response after starting HAART do not translate into a decrease in mortality. This study, which analyzed more than 20,000 antiretroviral-naïve individuals starting HAART in 1995–2003, indicated that despite improved initial HIV virological control in patients taking HAART, in more recent years there were no significant improvements in early immunological response, no reduction in all-cause mortality and a significant increase in combined AIDS/AIDS-related death (May *et al.*, 2006). Third, the emergence of drug-resistant HIV variants remains a major barrier to the successful use of HAART in HIV-infected patients, with resistance to one drug often resulting in cross-resistance to many, if not all, others in the same class. In addition, the rise in the incidence of drug-resistant variants among newly infected patients also represents a formidable challenge for clinicians (Clotet, 2004). Fourth, and perhaps the most important, while HAART significantly reduces the levels of viral RNA in plasma and lymphoid tissues, cessation of even prolonged HAART regimens results in viral load rebound to pretherapy levels, indicating the inability of therapy to eradicate HIV-1 infection (Chun and Fauci, 1999; Chun *et al.*, 1999; Persaud *et al.*, 2003; Siliciano *et al.*, 2003). This failure has been attributed to the presence of a long-lived, stable population of latently infected cells that are not eliminated by the antiviral treatment. Several of these cells are long-lived memory T cells that have an integrated proviral DNA that is kept in a transcriptionally silent state (Marcello *et al.*, 2004). The persistence of latent HIV-1 reservoirs is the principal barrier in the complete eradication of HIV-1 infection in patients by HAART at present.

In light of these considerations, and given the need for alternative therapies that might control HIV infection, it is not surprising that gene therapy has of late regained much popularity.

Available gene transfer technologies have been exploited to pursue the gene therapy of HIV infection according to different strategies, as shown in Table I. As early as 1988, D. Baltimore put forward the concept of "intracellular immunization" as a strategy to inhibit the replication of HIV-1 at the cellular level (Baltimore, 1988). There are several ways in which the cells can be rendered resistant to HIV-1 infection, which can be broadly categorized according to whether the specific targets are proteins of viral origin, viral nucleic acids, or cellular proteins that are essential to permit HIV-1 infection or sustain viral replication. An alternative strategy, proposed in the early days of HIV gene therapy but scarcely pursued, is to selectively induce cell death or expression of antiviral genes on cell infection with HIV-1. Finally, a completely different approach is to target the immune system. This goal can be achieved by either vaccinating patients against HIV

TABLE I Strategies for Gene Therapy of HIV-1 Infection

"Intracellular immunization" by targeting viral proteins	Intracellular antibodies RNA or DNA decoys Transdominant mutants
"Intracellular immunization" by targeting viral RNA	Antisense RNAs or DNAs Ribozymes siRNAs
"Intracellular immunization" by targeting cellular factors essential for HIV infection or HIV replication	Expression of cellular factors that restrict HIV replication (e.g., rhesus monkey TRIM5α) Inhibition of cellular factors essential for HIV infection (e.g., CCR5)
Selective activation of suicidal or antiviral genes on HIV-1 infection	For example, LTR-TK, LTR-IFN
Activation of the host immune system	Vaccination against HIV proteins Genetic modification of $CD8^+$ cells

proteins or selectively activating a cytotoxic response against HIV-1 infected cells by the transfer, into lymphocytes, of genes that direct the immune response against virus-infected cells.

This chapter will specifically focus on the attempts made so far to render T-cells resistant to HIV-1 infection and, more precisely, on the experience gained to date from the results of clinical trials (Table II). The purpose is to discuss the limits of the experimental approaches so far implemented, to describe the current status of research, and to critically highlight the advancements that are required before gene therapy of HIV-1 infection might eventually become clinically successful.

III. Gene Therapy of HIV Infection: Lessons from Early Clinical Trials

From the mid-1990s, a variety of approaches have been described for the suppression of HIV-1 infection or replication in cultured cells (Bridges and Sarver, 1995). These have included the expression of (1) intracellular antibodies to viral proteins; (2) antisense RNAs that inhibit reverse transcription, processing, and translation of HIV-1 RNAs; (3) mutant HIV structural or regulatory genes with dominant repressor activity (including *Rev*, *Gag*, and *Tat*); (4) RNA decoys that inhibit HIV-1 transcription [multimeric transactivation response element (TAR)]; and processing [multimeric Rev-response element (RRE)]; and (5) ribozymes to catalytically cleave and thus inactivate the various HIV-1 RNA species. A comprehensive discussion

TABLE II Major Gene Therapy Clinical Trials Aimed at Rendering $CD4^+$ Cells Resistant to HIV-1 Infection

Therapeutic gene	*Vector*	*Target cells*	*References*
RevM10 (dominant negative Rev mutant)	Plasmid transfection using gold microparticles	$CD4^+$ T lymphocytes	Woffendin *et al.*, 1996
	Retrovirus	$CD4^+$ T lymphocytes	Ranga *et al.*, 1998
		$CD34^+$ bone marrow cells	Podsakoff *et al.*, 2005
		Mobilized allogenic $CD34^+$ cells from peripheral blood	Kang *et al.*, 2002
RevM10 and/or *RevM10* plus antisense *TAR*	Retrovirus	$CD4^+$ T lymphocytes from HIV-negative identical twins	Morgan and Walker, 1996; Morgan *et al.*, 2005
RRE decoy	Retrovirus	$CD34^+$ bone marrow cells	Kohn *et al.*, 1999
Antisense RNAs against *TAR* and *Tat*/*Rev* (HGTV43)	Retrovirus	$CD34^+$ bone marrow cells	
Long antisense against env, driven by the HIV-1 LTR (VRX496)	Lentivirus	$CD4^+$ T lymphocytes	Levine *et al.*, 2006; MacGregor, 2001
Hairpin ribozyme against HIV-1 U5 leader sequence	Retrovirus	$CD4^+$ T lymphocytes	Looney *et al.*, 1998
Hammerhead ribozyme targeted to *Tat* and *Rev*	Retrovirus	$CD34^+$ bone marrow cells	Krishnan *et al.*, 2002; Michienzi *et al.*, 2003; Zaia, 2003
		$CD34^+$ bone marrow cells after myeloablation	Krishnan *et al.*, 2002; Michienzi *et al.*, 2003; Zaia, 2003
Hammerhead ribozyme against the translation initiation region of tat (Rz2, OZ1)	Retrovirus	$CD4^+$ T lymphocytes from HIV-negative identical twins	Macpherson *et al.*, 2005
		Mobilized $CD34^+$ cells from peripheral blood	Amado *et al.*, 1999, 2004
Gp41-derived peptide blocking fusion (M87o)	Retrovirus	$CD4^+$ T lymphocytes	Egelhofer *et al.*, 2004
TAR decoy RNA, siRNA against *Tat* and *Rev*, ribozyme against cellular *CCR5*	Lentivirus	$CD34^+$ bone marrow cells	Li *et al.*, 2005

of these *in vitro* approaches is beyond the scope of this review and can be found elsewhere (Macpherson *et al*., 1999; Strayer *et al*., 2005; Wolkowicz and Nolan, 2005).

Taken together, the work performed in cell culture systems has indicated overall that the goal of rendering cells resistant to HIV-1 infection by gene transfer is attainable. A major challenge, however, has been the adaptation of these genetic strategies to a clinical setting.

A. Clinical Trials Using Dominant Negative Forms of the HIV-1 Rev Protein

Perhaps the most investigated gene for gene therapy of HIV-1 is the one coding for a dominant negative mutant of *Rev*. Rev is a 116-amino acid viral protein essential for viral replication, which is translated from a fully spliced mRNA expressed early in viral infection. The protein shuttles between the nucleus and the cytoplasm, and acts posttranscriptionally to mediate the cytoplasmic export of unspliced (≈9 kb) and singly spliced (≈4 kb) viral RNAs, which encode the virion proteins Gag, Pol, and Env. This function requires the direct and highly specific binding of Rev to the RRE present in these unspliced RNAs. A mutant protein, RevM10, which blocks Rev function has been described (Malim *et al*., 1989). This mutant, which bears two amino acid substitutions in the highly conserved leucine-rich region of the protein, is still able to bind RRE RNA, but no longer binds the Crm-1 nuclear export factor. Since it still multimerizes with other Rev monomers, RevM10 acts as a dominant negative inhibitor of Rev and blocks transport into the cytoplasm of the incompletely spliced HIV-1 transcripts. When delivered by retroviral vectors to T cell lines and primary bone marrow cells, RevM10 acts as a powerful suppressor of viral replication (Bauer *et al*., 1997; Bevec *et al*., 1992; Malim *et al*., 1992).

An early clinical study assessed the feasibility of gene therapy of HIV-1 infection by delivering RevM10 to $CD4^+$ T cells from three HIV-infected individuals by plasmid transfection using gold microparticles, followed by *in vitro* expansion and reinfusion of the transduced cells back to the patients (Woffendin *et al*., 1996). The cells expressing RevM10 showed a transient four- to fivefold selective survival advantage; however, they were not detectable after 8 weeks from infusion. To determine whether more durable engraftment could be achieved, transfer of the *RevM10* encoding gene was performed in an additional three patients using murine retroviral vectors and improved conditions for T cell stimulation. After these procedures, the cells expressing RevM10 were detectable for an average of 6 months after reinfusion into the patients, compared with 3 weeks with control cells, thus showing that the expression of this mutant effectively prolonged cell survival (Ranga *et al*., 1998). A potential obstacle to the persistence of cells expressing viral proteins *in vivo* is the development of host immune responses against

the cells expressing these proteins. However, though RevM10 has been shown to be immunogenic in mice (Chan *et al.*, 1998), no apparent cellular immune response against the transduced cells was detected in these patients.

The selective advantage conferred by RevM10 to the expressing $CD4^+$ cells has also been confirmed by an additional experimentation involving retrovirus-mediated gene transfer into $CD34^+$ human hematopoietic stem cells (HSCs) in two HIV-1-infected children (Podsakoff *et al.*, 2005). In one of these patients, despite the extremely low frequency of gene-modified cells in the circulation beyond the first 3 months after transduction (≤ 1 every 1×10^6 cells), modified clones reappeared concomitant with a rise in the HIV-1 viral load during a period of nonadherence to the antiretroviral regimen. This observation reinforced the notion that active HIV-1 replication might impart selective pressure to gene-modified cells leading to their expansion *in vivo*.

Additional clinical trials exploiting the dominant negative properties of Rev mutants have been performed by infusing $CD4^+$ T lymphocytes transduced with a retroviral vector containing negative dominant Rev in combination with an antisense molecule that inhibits viral replication by binding to the TAR RNA. The protocol involved isolating $CD4^+$ T lymphocytes from HIV-negative identical twins of HIV-positive patients, *ex vivo* transduction followed by infusion into the HIV-positive sibling (Morgan and Walker, 1996). Results of 19 separate treatments, with follow-up over 3 years, demonstrated that genetically modified cells expressing the resistant genes could be detected for longer periods of time compared to T cells engineered with a marker gene, albeit at a very low level [100–400 cells every million total peripheral blood mononuclear cells (PBMCs)]. Of interest, the ratio of therapeutic to control vector-containing cells markedly increased in one patient who discontinued HAART treatment, further supporting the rationale of the experimentation that HIV-1 infection imposes a selective pressure on resistant cells (Morgan *et al.*, 2005).

Collectively, the results obtained by these early clinical trials are encouraging, since they confirm that Rev-dominant mutants might also confer resistance to HIV-1 infection *in vivo*. Further improvements are however essential to increase the efficiency of gene transfer to *ex vivo*-cultured hematopoietic progenitors, a problem that is more generally hampering the success of several clinical experimentations of gene therapy of hematopoietic progenitors. This problem might be partially overcome by expressing dominant negative Rev from HIV-1-based lentiviral vectors (Bahner *et al.*, 2007; Mautino and Morgan, 2002), as discussed later.

B. Clinical Trials Using the RRE Decoy

The potency of RevM10 to suppress HIV-1 replication clearly indicates that Rev function is essential for HIV-1 replication. Consistent with this conclusion, the overexpression of the RRE sequences as part of the

transcript from the viral long terminal repeat (LTR) of a retroviral vector leads the inhibition of HIV-1 replication in both T lymphocytes and the progeny of transduced $CD34^+$ hematopoietic progenitor cells (Bauer *et al.*, 1997). Following these observations, four HIV-1-infected children and adolescents underwent bone marrow harvest from which $CD34^+$ cells were isolated and transduced by a retroviral vector carrying this RRE decoy gene. The cells were reinfused into the subjects, without complications. However, also in this case, gene-containing leukocytes in the peripheral blood were seen only at a low level (1–3 cells every 1×10^5 peripheral blood cells) and only in the first month following cell infusion (Kohn *et al.*, 1999).

C. Clinical Trials Using Antisense RNAs

Over the last several years, different investigators have reported the efficacy of intracellularly expressed antisense RNA molecules targeted against different HIV-1 genes in preventing HIV-1 infection. In addition, retrovirus-mediated delivery of antisense genes targeting tat and rev to rhesus macaque $CD4^+$ T lymphocytes using retroviral vectors followed by the infusion of the transduced cells in the animals was found to significantly reduce viral load after challenge with SIVmac239 (Donahue *et al.*, 1998). One efficient way to express such antisense RNAs is as fusions to the U1 snRNA transcript, an essential component of the cellular splicing machinery which is abundantly and constitutively expressed in all cells (Liu *et al.*, 1997). A retroviral vector expressing three antisense RNA sequences targeting TAR and two sequences in the *tat/rev* genes as fusions to U1 was used to deliver these genes into $CD34^+$ peripheral blood progenitor cells of five HIV-infected individuals. These patients were subsequently engrafted with the modified cells without bone marrow ablation. Similar to the previously discussed studies, persistence of the resistant cells could be detected for several years after transduction, albeit their frequency has remained below the threshold required to provide significant therapeutic benefit. These approaches are now exploiting the possibility of improving the efficiency of gene transfer using either retroviral vectors after bone marrow conditioning, or by the more efficient delivery of antisense genes to bone marrow HSCs using lentiviral vectors (see below).

D. Clinical Trials with Ribozymes

Another class of molecules that has been widely utilized over the last several years to inhibit HIV-1 replication by cleaving the viral genome and transcripts is ribozymes (Haseloff and Gerlach, 1988). These RNA enzymes have the potential to act at several stages in the HIV infectious cycle, including the initial entry of genomic viral RNA into the target cell, during the transcription of genomic RNA molecules, prior to and during

translation of mRNA to viral proteins, and prior to encapsidation of the genomic RNA. The cleavage of HIV RNA by ribozymes at any of these stages can significantly decrease or block intracellular viral replication (Macpherson *et al*., 1999). When choosing a ribozyme, due to the sequence variation among HIV-1 isolates and the rapid mutation rate in response to antiretroviral treatment, it is imperative to select target sites that are critical for viral replication and highly conserved in sequence between clades. Over the last several years, a number of hammerhead or hairpin ribozymes have been described that fulfill these criteria, and have been variously described to reduce HIV-1 replication in cell culture (reviewed in: Macpherson *et al*., 1999; Rossi, 1999; Sarver *et al*., 1990).

In the early 1990s, it was first demonstrated that a gene encoding a hairpin ribozyme targeted to the HIV-1 U5 leader sequence conferred resistance to HIV-1 infection when delivered to T cell lines and primary lymphocytes using a retroviral vector (Yu *et al*., 1994). Based on these results, a phase I clinical trial was initially conducted on six HIV-infected individuals by transducing their peripheral blood lymphocytes with a retrovirus expressing this ribozyme (Wong-Staal *et al*., 1998). The initial results of this trial showed a modest survival advantage for cells expressing the ribozyme (Looney *et al*., 1998). Other initial attempts at exploiting anti-HIV-1 ribozymes for gene therapy of HSCs have also been quite disappointing. Five healthy HIV-positive subjects who received autologous $CD34^+$ cells transduced with a retroviral vector expressing ribozymes targeted to tat and rev showed minimal transient engraftment of marked cells (Michienzi *et al*., 2003; Zaia, 2003). Slightly better were the results obtained in five other AIDS lymphoma patients, who were reinfused with $CD34^+$ cells modified using the same retrovirus after myeloablative treatment. These patients showed a significant increase in gene-marked cells posttransplant. However, the durability of this engraftment was short-lived, as indicated by the loss of observable gene marking 6 months posttransplant, most likely indicating transduction of an already committed progenitor cell population (Krishnan *et al*., 2002).

More encouraging results have been obtained by using another ribozyme-expressing retroviral vector. Rz2 is a hammerhead ribozyme targeting the conserved translation initiation region of the HIV-1 tat gene (Sun *et al*., 1995). This ribozyme was cloned into the 3′ untranslated region of the *neoR* gene, which was expressed under the control of the SV40 promoter in the context of a murine retroviral vector (Macpherson *et al*., 1999). Phase I clinical trials involving the delivery of this vector were conducted by transducing $CD4^+$ T lymphocytes in identical twins discordant for infection or autologous mobilized peripheral blood $CD34^+$ hematopoietic progenitors, which were reinfused without myelosuppression (Amado *et al*., 1999). In both trials, separate populations of cells were transduced with either a retroviral vector containing the ribozyme or the vector alone. Equal numbers

of the two transduced cell types were then reintroduced into the recipient patients, in order to monitor the survival of anti-HIV-1 ribozyme-expressing cells. After gene transfer into $CD4^+$ T lymphocytes, both retroviral vectors were detected in PBMCs for up to 4 years, without evidence of immune elimination of the neoR-expressing cells or silencing of gene expression (Macpherson *et al.*, 2005). The phase I clinical trial conducted by gene transfer in $CD34^+$ cells from 10 patients indicated that the transgene could be detected up to 30 months in multiple hematopoietic lineages, with a frequency of $1/10^4$ to $1/10^5$ of hematopoietic cells analyzed, including naïve T cells. Since the $CD4^+$ cells expressing the ribozyme had no selective advantage, the patients being on HAART, the number of transduced cells remained low and no significant enrichment of the Rz2-expressing cells could be detected over the control cells (Amado *et al.*, 2004). Based on these relatively encouraging results, a midsize (70 patients) phase II clinical trial has now been started in both the USA and Australia, in which selective pressure is applied to the transduced cells by stopping patients' antiretroviral therapy (http://www.clinicaltrials.gov/ct/show/NCT00074997).

IV. Gene Therapy of HIV Infection: Current Developments

From the review of the clinical trials conducted so far, it can be safely concluded that (1) the eventual success of gene therapy for HIV-1 infection by the induction of intracellular resistance to HIV-1 will require the prolonged expression of the therapeutic gene in order to select, over year-long periods, a sufficient number of resistant $CD4^+$ T lymphocytes; (2) that this task will be only feasible when improved conditions for gene transfer of $CD34^+$ hematopoietic progenitors are developed. In addition, improvements are clearly needed in terms of therapeutic gene choice. The following section reviews some of the recent developments in these areas. In particular, it discusses the problems related to gene transfer into HSCs, the entry into the gene therapy arena of gene transfer vectors based on HIV-1, the possibility of exploiting RNAi for antiviral purposes, and, finally, the identification of cellular genes as possible targets for HIV-1 gene therapy.

A. Gene Transfer to Hemapoietic Progenitors

In the last decade, there have been several monogenic diseases for which seminal gene therapy trials have been conducted by the γ-retrovirus-mediated gene transfer of normal cDNAs into autologous $CD34^+$ HSCs (Kohn *et al.*, 2003). The overall results of these trials have indicated that *ex vivo* gene transfer with γ-retroviruses targets a population of already committed hematopoietic progenitors, with limited clonal potential, which disappear from

the circulation in a few weeks or months. In contrast, the number of truly multipotent HSCs able to sustain long-term hematopoiesis that are transduced is very low. Although the progeny of these transduced cells can be observed in the peripheral blood for over a decade from gene transfer (Muul *et al*., 2003), their frequency does not usually exceed 0.1% of the total cell population. The low frequency of transduction of the multipotent HSC by γ-retroviral vectors is most likely due to the property of this cell to be out of the cell cycle and to the intrinsic incapacity of retroviral vectors to reach the cell nucleus in the absence of cell division. For reasons still unknown, all cytokine cocktails that activate *ex vivo* proliferation of HSC also determine their lineage commitment: although transduced at an efficiency close to 100% *ex vivo*, these committed cells have a limited self-renewal capacity once reinfused into the patients.

A fortunate condition that overcomes this problem is when transduction itself confers a selective advantage to the HSC, as reported to occur after gene transfer of the cDNA coding for the common γ-chain of the interleukin receptors in children with X-linked severe combined immunodeficiency (X-SCID) (Cavazzana-Calvo *et al*., 2000). Under this condition, the corrected HSCs and their progeny possess a remarkable growth advantage over the nontransduced cells, and are rapidly selected and expanded over a period of a few weeks once reinfused into the patients. In the case of HIV-1 infection, however, it might be expected that selection driven by viral infection might operate on mature $CD4^+$ T cells bearing HIV-resistance genes, but not at the level of the HSC and its immediate progeny. As a consequence, transduction imparts no survival advantage to $CD34^+$ cells.

At least two other genetic disorders, adenosine deaminase deficiency (ADA) (Aiuti *et al*., 2002) and chronic granulomatous disease (CGD) (Ott *et al*., 2006), have been successfully treated by gene therapy of the HSCs by ablating the recipient's bone marrow by chemotherapy before the reinfusion of transduced cells. In both trials, nonmyeloablative conditioning was induced in the patients receiving the retrovirus-modified cells by using busulfan, a relatively manageable myelosuppressive drug. The success of this approach indicates that the transduced HSC might find a better possibility to expand in the context of a partially ablated bone marrow, and suggest that a similar strategy might significantly improve the outcome of HIV gene therapy clinical trials as well. Indeed, a phase II clinical experimentation entailing the infusion of HSCs transduced with the same retroviral vector expressing antisense RNAs fused to the U1 snRNA transcripts described earlier (Liu *et al*., 1997), but after bone marrow conditioning, will prove whether this paradigm will also hold true for HIV patients.

One important question that needs to be thoroughly addressed is whether genetically modified HSCs can reconstitute the immune system in adults. Although studies have demonstrated that the adult uninfected thymus maintains the ability to support T-lymphopoiesis (Jamieson *et al*., 1999;

Poulin *et al*., 1999), after transplantation of HIV-negative patients, functional recovery of lymphoid and immune effectors cells occurs gradually, and reconstitution of normal humoral and cellular immunity may take a year or more (Guillaume *et al*., 1998). In uninfected individuals, T-cell reconstitution takes place by either peripheral expansion of the already existing T cell pool or by renewal of thymopoiesis. In HIV-infected individuals transplanted with HSCs carrying HIV-resistant cells, thymopoiesis is absolutely required to generate a nonskewed repertoire of HIV-resistant naïve T cells. During thymocyte development, rearrangement of the T cell receptor gene leads to the excision of circular DNA fragments from genomic DNA, among which signal joint T-cell receptor excision circles (sjTRECs). These products are stable, unique to T cells and are not duplicated during mitosis, which means that they are diluted out with each cellular division. Therefore, the levels of sjTRECs in peripheral blood T cells has been extensively used as a marker for thymic function after both allogenic and autologous stem cell transplantation in noninfected individuals (Chen *et al*., 2005; Talvensaari *et al*., 2002; Weinberg *et al*., 2001). In HIV-infected patients, thymic function was found to be depressed but still present, and the levels of sjTREC was found to correlate with the success of HAART (Douek *et al*., 1998; Nobile *et al*., 2004). In addition, bone marrow transplantation was effective in HIV patients with lymphoma (Gabarre *et al*., 2000; Krishnan *et al*., 2005). Taken together, these observations indicate that immune reconstitution after transplantation of genetically modified hematopoietic progenitors is an attainable objective. This conclusion is also supported by the observation that gene-modified naive T-lymphocytes could be detected in patients who underwent HSC gene transfer using a retroviral vector expressing the anti-HIV Rz2 ribozyme (Amado *et al*., 2004).

Two "successful" gene therapy trials, those for X-SCID (Cavazzana-Calvo *et al*., 2000) and CGD (Ott *et al*., 2006), have dramatically brought into evidence an additional problem that needs to be considered by all the experimentations aimed at transferring genes into HSCs using retroviral vectors, namely that of insertional activation of protooncogenes due to random retroviral insertion into the genome. In both these trials, a murine leukemia virus-derived replication incompetent γ-retroviral vector was used to infect purified $CD34^+$ cells with the therapeutic gene being expressed by the vector LTR. In the French X1-SCID trial, three patients developed leukemia between 2 and 3 years after the infusion of transduced cells due to the activation of the LMO2 protooncogene (Hacein-Bey-Abina *et al*., 2003a,b). In the CGD trial, the insertional activation of the *MDS1-EVI1* genes may have contributed to clinical benefit but also led to a clonal myeloproliferation resembling a preleukemic state (Ott *et al*., 2006).

Ample evidence now indicates that retroviral integration is not random, as previously believed, with as many as 20–25% of all integration events occurring within 10 kb of gene promoter elements (Laufs *et al*., 2003;

Wu *et al*., 2003). In addition, cells bearing insertions into or near genes involved in cell proliferation or antiapoptosis display preferential engraftment and survival (Kustikova *et al*., 2005). In the case of the X1-SCID trial, it still remains uncertain whether, in addition to these characteristics common to all retroviral constructs, functional cooperation between the transduced and overexpressed γ-chain gene and the LMO2 oncogene might have further favored the expansion of the clonal population bearing the retrovirus inserted into the LMO2 locus (Dave *et al*., 2004; Thrasher *et al*., 2006; Woods *et al*., 2006).

Various modifications of the vector backbone have recently been described, which might limit the transcriptional activation of cellular genes on retroviral integration. These include the use of insulators or enhancer-blocking elements, the inclusion of a suicide gene in the vector backbone that might be turned on upon inappropriate proliferation of the transduced cells, or, most importantly, the construction of self-inactivating (SIN) vectors, in which the 5′ LTR U3 region becomes deleted on reverse transcription in the target cells and transcription becomes driven by an internal promoter (Nienhuis *et al*., 2006).

Although at this moment it is still unclear to what extent the oncogenic potential of insertional mutagenesis by γ-retroviral vector-mediated HSC transduction might specifically impact gene therapy of HIV-1 infection, several of these ameliorations in retroviral vector design might well apply to this field of application.

B. Gene Therapy Using Lentiviral Vectors

Viral vectors based on HIV-1 have entered the gene therapy arena about 10 years ago (Naldini *et al*., 1996) and have rapidly elicited broad interest especially for their property to transduce quiescent cells, including the HSC (Miyoshi *et al*., 1999; Sutton *et al*., 1998). Multiple viral proteins, including Matrix, Vpr, and Integrase confer to the wild-type HIV-1 pre-integration complex the capacity to enter the nucleus of nonreplicating cells (Fassati, 2006). While Vpr is not included in the third generation packaging systems for lentiviral vectors, both Matrix and Integrase are essential components of these vectors and are sufficient to mediate their nuclear import.

Several issues concerning both the efficiency and the safety of these vectors still remain to be elucidated. These include the definition of the optimal vector design, the exact understanding of their mutagenic potential, and the characterization of their propensity of being silenced over time in different tissues, together with safety concerns regarding their clinical utilization (Chang and Sadelain, 2007). These safety concerns mainly relate to the possibility of recombination of a lentiviral vector into a replication-competent lentivirus (RCL) that might represent a novel pathogen, and the possibility of insertional oncogenesis after vector integration into the host genome, as specifically discussed later.

These perceived risks have significantly slowed down the introduction of these vectors to the clinics. As recently as 2003, the first lentiviral vector trial was initiated for gene therapy of HIV infection. The vector expressed a long (937 nt) antisense RNA against the HIV envelope gene from the HIV LTR, and was used to transduce autologous $CD4^+$ T cells. This phase I trial was conducted in five subjects with chronic HIV infection who had failed to respond to at least two antiviral regimens by a single infusion of gene-modified cells (MacGregor, 2001). The results obtained in this trial showed prolonged engraftment with lentivirus-modified T cells in three of the patients for at least 1 year after infusion, albeit at low levels. No statistically significant anti-HIV effects were observed, but one patient developed a sustained decrease in viral load (Levine *et al*., 2006). Two follow-on studies are presently underway to evaluate the potential of this approach when given in repeated doses and in the context of structured treatment interruption (STI) (www.clinicaltrials.gov).

Two additional lentivirus vector-based trials are planned, one entailing gene transfer of RevM10 in HSCs after partial bone marrow ablation (Bahner *et al*., 2007), and another delivering multiple anti-HIV genes [RNA decoy for TAR, RNAi against Tat and Rev, and ribozyme against chemokine (C-C motif) receptor 5 (CCR5); Li *et al*., 2005]—see also below.

As for γ-retroviruses above, a critical issue that has emerged from the recent gene therapy clinical trials for X-SCID and CGD is related to the oncogenic potential of these vectors after gene transfer into HSCs. How does the concern for insertional mutagenesis specifically apply to gene therapy using lentiviral vectors? While no study has yet systematically addressed this issue, at least two considerations suggest that the oncogenic potential of lentiviruses might be much more attenuated. First, the pattern of integration of HIV-1 into the genome is different to that of γ-retroviruses. Both viruses are more likely to integrate near transcribed genes, but HIV-1 does not preferentially target the region surrounding the transcription start site, as does murine leukemia virus (MLV) (Wu *et al*., 2003), but integrates along the entire length of the gene (Schroder *et al*., 2002). This different behavior might render lentiviral vectors less prone to aberrantly activate transcription of the targeted gene. Second, cell transformation is a peculiar characteristic in the life cycle of γ-retroviruses (but not of HIV-1), which appears to be specifically related by the activity of the retroviral U3 promoter/enhancer region in specific cell types. The vector used in the French X1-SCID gene therapy trial was based on the MFG vector backbone, which contains the Moloney MLV LTR; gene expression in the CGD trial was driven by the Friend mink cell spleen focus-forming virus (SFFV) LTR. Both are transforming retroviruses that cause thymic lymphomas and eryrthroleukemia, respectively, in susceptible mice based on the activity of their LTRs in different cell types. In contrast, lentiviral vectors based on HIV-1 might either directly express the transgene from the HIV-1 LTR, which is almost

completely silent in the absence of the viral Tat protein and can be thus reactivated only on T cell or macrophage infection by HIV-1, or take advantage of a SIN configuration, in which therapeutic gene expression is driven by appropriate internal promoters that are only activated in differentiated cells and thus do not drive the undesired expansion of the transduced HSCs.

Lentiviral vectors, however, raise other specific safety concerns. A major one is the possibility of the formation of a vector-derived RCL that, by exploiting the property of the vesicular stomatitis virus G protein (VSV-G) envelope protein used for pseudotyping, might extend the host range of HIV-1 to a much broader repertoire of target cells. Another specific issue relates to the mobilization of lentiviral vectors *in vivo* on infection of the transduced cells by HIV-1. This possibility is a specific concern when using lentiviral vectors with intact LTRs, which might therefore exploit the viral proteins produced by the wild-type virus in trans for their own transcription and packaging. This event cannot occur with SIN vectors, in which the 5′ LTR U3 region is deleted and transcription is driven by an internal promoter. The earlier described clinical trial using a lentiviral vector in which transcription of an anti-env antisense was driven by the vector LTR has indeed indicated that self-limiting mobilization of the vector occurred in four of five treated patients (Levine *et al.*, 2006). In this respect, however, it should be considered that, although vector mobilization *in vivo* to nontarget tissues may have adverse safety consequences, mobilization of the anti-HIV therapeutic gene into uninfected $CD4^+$ cells might be beneficial, since it could amplify the desired antiviral effects (Manilla *et al.*, 2005).

C. RNA Interference as a Therapeutic Tool

The process of double-stranded RNA-mediated RNAi has been originally discovered by Fire and Mello in the worm *C. elegans* as a powerful mechanism of suppression of gene expression (Fire *et al.*, 1998). The observation that RNAi also occurs in mammalian cells as part of a larger network of RNA silencing mechanisms that share common pathways (Sharp, 2001; Tuschl, 2002), has rapidly prompted its possible utilization as a tool to combat viral infections by targeting the destruction of viral RNAs. In 2002, it was demonstrated that synthetic siRNAs against the cellular CD4 or the viral *Gag* gene inhibit HIV-1 infection (Novina *et al.*, 2002) and, most notably, that siRNAs against different regions of the HIV-1 genome could be generated inside the cells after transfection of plasmids expressing short hairpin RNAs (shRNAs) (Jacque *et al.*, 2002; Lee *et al.*, 2002). In subsequent years, a number of studies have extended these observations, by targeting various other regions of the HIV genome, by inserting shRNA-expression cassettes into viral vectors for improved gene delivery, and by expanding the repertoire of target sequences to cellular genes known to be required for efficient HIV-1 infection (reviewed in: Morris and Rossi, 2006; Rossi, 2006).

More generally, it now appears that the attempt to inhibit HIV infection by exogenous therapeutic siRNAs should deal with the complex interplay existing between HIV nucleic aids and the host cell machinery that regulates microRNA (miRNA) production and activity (Provost *et al.*, 2006). Accumulating evidence in fact indicates that the cellular miRNA-silencing machinery restricts HIV-1 replication on one side, while, on the other side, the virus has evolved ways to cope with this inhibition. First, on HIV-1 infection, the expression levels of several cellular miRNAs are significantly altered; some of these miRNAs are putatively involved in the regulation of cellular factors that are essential for HIV replication (Triboulet *et al.*, 2007). Second, the miRNA machinery directly generates miRNAs from the HIV-1 RNA itself, which regulate HIV-1 infection (Bennasser *et al.*, 2004; Omoto and Fujii, 2005). Third, inhibition of Drosha (required for primary miRNA processing in the nucleus to generate 60–70 nt-long miRNA precursors) or Dicer (which, in the cytoplasm, activates miRNAs by generating miRNA:miRNA* duplexes and is then incorporated into effective miRNA-containing ribonucleoprotein complexes) significantly increases HIV-1 replication, further indicating that the miRNA pathway contributes to the suppression of HIV replication (Triboulet *et al.*, 2007). Fourth, overexpression of Tat attenuates silencing of reporter genes when this is induced by short hairpin (shRNAs) but not by siRNAs, since the protein directly inhibits Dicer (Bennasser *et al.*, 2005). Any possible gene therapy treatment aimed at inhibiting HIV-1 infection by RNAi should evidently deal with this intricate pathway of reciprocal regulation as well as evade the evolutionary mechanisms that the virus has evolved to escape inhibition by cellular miRNAs.

One of the crucial concerns that needs to be addressed in gauging the efficacy of RNAi for the therapy of HIV infection is the possibility of the emergence of escape mutants. Indeed, HIV-1 has been shown to easily evade RNAi by the selection of mutants encoding the same viral proteins but with silent mutations impairing siRNA recognition (Das *et al.*, 2004; Sabariegos *et al.*, 2006) or by evolving alternative structures in its RNA genome that occlude the siRNA binding site (Westerhout *et al.*, 2005). This problem might be overcome by the simultaneous expression of several siRNAs against multiple targets or, more efficiently, by targeting a cellular gene (Cullen, 2005), such as CCR5; see also below.

Besides the generation of escape mutants, another recent concern is related to the possibility that the unregulated expression of shRNAs might be toxic for the expressing cells. Recent work has indicated that the long-term expression of sustained high levels of shRNAs in livers of adult mice leads to liver failure and death, possibly due to the saturation of the normal miRNA machinery of the cells (Grimm *et al.*, 2006). Analogous conclusions were also obtained by transferring an antiCCR5 shRNA in T-lymphocytes using various promoters (An *et al.*, 2006). Together these results clearly indicate that it is imperative that one of the carefully evaluated variables in

developing an RNAi-based gene therapy strategy for HIV infection is the level of expression of the therapeutic gene and its long-term effect on cell viability.

D. Targeting HIV-1 Internalization

Most of the earlier described therapeutic genes (antisense, ribozymes, siRNAs, intracellular antibodies, decoys, and others) block HIV replication after the cell has internalized the virus and, most likely, after integration of the provirus into the host cell genome. For example, a recent study has clearly indicated that siRNAs targeting HIV RNA have no access to the incoming HIV genome, probably due to inaccessibility to the RNAi machinery (Westerhout *et al.*, 2006). One attractive possibility would therefore be to render the cells refractory to HIV infection by directly impeding its actual infection.

One drug that has recently entered the market is enfuvirtide, commonly known as T20 (Lalezari *et al.*, 2003). T20 derives from a 26-amino acid peptide from the C-terminus of HIV-1_{HXB2} gp41 (C36 peptide), which blocks HIV entry by inhibiting the conformational changes needed for fusion of the viral envelope with the cellular membrane (Eckert and Kim, 2001). For a gene therapy approach, this peptide was modified with an anchor protein for cell surface expression, and further optimized for reduced immunogenicity and improved expression and stability; the final version of the construct (called M87o) was expressed using a retroviral vector (Egelhofer *et al.*, 2004). Transduced cells express this peptide on their surface, a strategy that allows the attainment of a sufficiently high local concentration to inhibit fusion of the viral envelope to the cell membrane, exactly as T20 does. A pilot clinical trial in 10 patients with late stage HIV disease was performed by infusion of $CD4^+$ T cells transduced with the retroviral vector. Initial results from this trial indicated that the approach was safe and that enrichment for the transduced cells was detectable in the peripheral blood of some of the patients, although no changes in viral load were observed. One major concern about the clinical utilization of M87o stems from the observation that T20 elicits the rapid emergence of resistant viruses (Wei *et al.*, 2002), and therefore it needs to be used in combination with other antiretroviral drugs. It is therefore likely that the success of M87o will depend on its utilization in the context of multistrategy gene therapy.

Further in respect to viral entry, one of the most striking discoveries in the HIV research field has been the observation that individuals with homozygous deletions in the CCR5 chemokine receptor gene are genetically resistant to HIV-1 infection, independent of the route of transmission (Huang *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). Most importantly, these individuals do not appear to be associated with clinical conditions, suggesting that the biological function of CCR5, in contrast to that of CXCR4, is compensated

by other chemokine receptors, probably due to the redundancy of the chemokine family (Proudfoot, 2002). These observations demonstrated both the critical importance of CCR5 for HIV-1 infection and highlighted the dispensable nature of its function, thus suggesting that inactivation of CCR5 in lymphocytes or stem cells might be of therapeutic value.

Inactivation of CCR5 expression or function has been attempted by a variety of means, which include peptides derived from the natural ligands regulated upon activation, normal T-cell expressed, and secreted (RANTES) and macrophage inflammatory protein-1α (MIP-1α), chemical drugs, antisense peptide nucleic acids, as well as by different genetic approaches for the phenotypic knock down of the protein, which can be exploited by gene therapy. These include the delivery of both proteins and nucleic acids. The former category includes the utilization of anti-CCR5 intracellular antibodies (Steinberger *et al.*, 2000), dominant negative mutants (Luis Abad *et al.*, 2003), and intrakines (modified RANTES and MIP-1α CCR5 ligands targeted to the endoplasmic reticulum, which block the surface expression of newly synthesized CCR5; Yang *et al.*, 1997). Among the therapeutic nucleic acids, several groups have described the resistance to HIV-1 infection of cell lines treated with anti-CCR5 ribozymes (Bai *et al.*, 2002; Goila and Banerjea, 1998; Gonzalez *et al.*, 1998; Li *et al.*, 2003), antisense (Qureshi *et al.*, 2006) or, more recently, siRNAs (Anderson and Akkina, 2005; Arteaga *et al.*, 2003; Lee *et al.*, 2003; Martinez *et al.*, 2002; Qin *et al.*, 2003).

A major concern in using this strategy to block HIV-1 entry is the potential of selective pressure on the virus to use other coreceptors, that is, CCR1, CCR2b, or CCR3, or to convert more rapidly toward X4-tropic HIV-1, especially considering that clinical experimentation will first be conducted in patients with late-stage disease. In addition, pharmaceutical companies have CCR5-inhibiting drugs in clinical trials, which might contribute to the emergence of mutant viruses that use alternate receptors. Given these considerations, it is likely that successful gene therapy of HIV-1 infection might eventually take advantage of CCR5 down-modulation, but in the context of a multitargeted strategy.

V. Gene Therapy for HIV Infection: Where Are We Heading?

From the earlier reported survey of the clinical experience gained over the last 10 years, it can be generally concluded that we are still far from having developed a safe and successful way of treating HIV-1 infection by gene therapy. However, similar to most gene therapy applications, the information gained in this first round of experimentation now allows the design of improved strategies that might overcome the problems so far encountered. Most likely, the road to success will rely on the combination of several of the different strategies so far developed.

As has emerged virtually from all clinical studies, one of the critical issues that hampers success of *ex vivo* gene therapy is the number of cells that can be engineered in the laboratory and then reinfused into the patients. Several experimentations have indicated that active HIV infection (e.g., after HAART discontinuation) might itself impose selective pressure on HIV-resistant cells and favor their expansion. However, this pressure is only exerted on differentiated $CD4^+$ T cells and not on hematopoietic precursors, which are not susceptible to infection. In addition, immune reconstitution after HSC engraftment is slower in HIV patients. For all these reasons, it appears that, although gene transfer into HSCs is in principle superior in efficacy compared to gene transfer into peripheral blood $CD4^+$ T lymphocytes, the success of this procedure will strictly depend on the possibility of transducing a sufficiently high number of cells. The combined use of lentiviral vectors (which efficiently transduce resting bone marrow progenitors) with the adoption of myeloblative regimens (which favors engraftment of the *ex vivo* modified cells) will be likely to significantly increase the number of resistant $CD4^+$ T cells that will ultimately be generated *in vivo*. The recent CDG trial indicates that γ-retroviral vector-mediated gene transfer into autologous hematopoietic cells and transplantation under conditions of incomplete myeloablation are sufficient to establish >10% gene-modified myelopoiesis within a year posttransplant (Ott *et al.*, 2006). However, marking levels in T cells are unfortunately much lower in the absence of selective advantage (Aiuti *et al.*, 2002). It might thus be reasonable to expect that the utilization of lentiviral vectors will significantly improve this efficiency.

An additional interesting possibility is the incorporation in the vector construct of a gene that might confer a selective advantage to the HSC on drug treatment. An example of such a gene is a mutant of *O*(6)-methylguanine-DNA-methyltransferase (MGMT), a nuclear enzyme that reverses toxic and mutagenic lesions produced on guanines by DNA-alkylating agents such as bis-chloroethyl-nitrosourea (BCNU) and bezylguanine (BG) (Davis *et al.*, 1997) or their less toxic derivatives. Viral vectors expressing this gene together with HIV-resistance genes have been recently described to confer selective advantage to transduced cells in cell culture (Davis *et al.*, 2004; Schambach *et al.*, 2006).

An additional essential problem that gene therapy of HIV infection needs to overcome is the extraordinary capacity of HIV-1 to rapidly select for escape mutants. The solution to this problem will most likely derive from the simultaneous use of several therapeutic genes, which will combinatorially lower the probability of emergence of resistant variants. This is indeed the same principle that led to the development of efficacious pharmacological treatment using multiple drugs. For example, in cultured T cells, HIV-1 can escape from shRNA inhibition by mutating after just 25 days (Boden *et al.*, 2003), but infection is controlled for at least several months by using a combination of four shRNAs (ter Brake *et al.*, 2006). Long-term inhibition of HIV-1 infection

after transduction of both PBMCs (Li *et al.*, 2003) and primary hematopoietic cells (Li *et al.*, 2005) has been obtained by SIN lentiviral vectors expressing a triple combination of Pol III-U6 promoter-driven shRNA targeting the HIV-1 rev and tat mRNAs, a U6-transcribed nucleolar-localizing TAR RNA decoy, and a VA1-derived Pol III cassette that expresses an anti-CCR5 ribozyme. Such a strategy is currently being adopted in a clinical trial.

Another possibility that renders the emergence of viral escape mutants less likely is the inhibition of cellular genes that are essential for HIV-1 replication, rather than viral genes. A striking example of this possibility is the CCR5 chemokine receptor, as discussed earlier. In addition to CCR5, over the last few years a flourishing number of other host cell factors have been described that might represent specific targets or tools to render cells resistant to HIV-1 infection. Among several others (Wolkowicz and Nolan, 2005), novel strategies exploiting this information might include the delivery of the simian version of TRIM5α, a protein which, in contrast to its human counterpart, binds the HIV-1 capsid and interferes with the uncoating process, thus protecting human cells from productive infection (Sakuma *et al.*, 2007; Stremlau *et al.*, 2004), or the inhibition of TSG101, which is required for vacuolar sorting and efficient budding of HIV-1 progeny (Garrus *et al.*, 2001) or of cyclin T1, since this protein is an essential cofactor of HIV-1 Tat in bringing to the HIV-1 promoter the cyclin-dependent kinase 9 (CDK9) kinase, required for efficient viral transcription (Bai *et al.*, 2003). These strategies, however, are fraught with the problem that the vast majority of these potential targets are essential for several cell functions, and that interfering with their activity might significantly impair cell viability. Recent data indicate that even CCR5 inhibition, which is usually considered safe since individuals with homozygous inactivating mutations of the *CCR5* gene are apparently normal, might instead be harmful. In fact, genetic studies in both mice and humans have provided strong evidence that *CCR5* plays an important role in controlling infection with the West Nile Virus (WNV), a reemerging pathogen responsible for fatal encephalitis. Thus, blocking CCR5 could augment the risk of symptomatic WNV disease (reviewed in Lim *et al.*, 2006).

The question whether we should look for additional target genes or targeting tools beyond the existing ones deserves a final consideration. In general terms, it appears that there are distinct advantages to an RNA-based approach, including antisense, ribozymes, decoys, and siRNAs. In fact, heterologous RNA molecules expressed in cells are not immunogenic and multiple sequences on both HIV genome and cellular genes can be targeted simultaneously, thus addressing the issue of HIV resistance. However, some peptidic molecules might offer selective advantages over RNAs, since they might be capable of inhibiting earlier steps of the infectious HIV-1 cycle, such as cell entry, uncoating, reverse transcription, or integration. A class of molecules that still shows promise in this respect is that of intracellular antibodies (or intrabodies), which represent the antibody

single-chain variable fragments (scFV) selected against various HIV proteins. For the past 15 years, virtually every HIV-1 protein has been targeted by intrabodies, including structural proteins (Matrix, Nucleocapsid, and Envelope), enzymes (Integrase and Reverse transcriptase), and regulatory proteins (Tat, Rev, and Nef; reviewed in Lobato and Rabbitts, 2003; Rondon and Marasco, 1997). So far, progress in the clinics has been slow, possibly because of the relatively lower antiviral effect shown by these molecules when compared to other therapeutics. However, intrabodies remain an attractive option when protein half-life is long and, most importantly, when a protein has more than one protein-interaction domain, because it is possible to develop a reagent that prevents particular associations but spares others (Lobato and Rabbitts, 2003). This might turn out to be an especially interesting tool to selectively target some of the cellular proteins that are essential for HIV-1 replication, by preserving their cellular function while inhibiting their proviral activity.

In conclusion, what is the probability that a successful gene therapy strategy is developed in the next few years that might cure or control HIV infection? After considering both the experience gained in the clinical trails and the technological advances that are currently available, there is definitely room for a cautious optimism in answering this question. As for all the ambitious goals, we should be aware that the progress in this field will continue to be slow and made out of small, incremental steps. However, we absolutely need to be perseverant, since gene therapy of HIV infection, despite its difficulty, still represents the most likely option to overcome the intrinsic limit of drug therapy in eradicating the infection and the low probability that a therapeutic vaccine might soon become available.

Acknowledgments

The author wishes to thank M. Lusic, L. Manganaro, and C. Vardabasso for critical reading of the manuscript and S. Kerbavcic for superb editorial assistance. The support of a grant from the Istituto Superiore di Sanita' Italy for AIDS research is gratefully acknowledged.

References

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., *et al.* (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**, 2410–2413.

Amado, R. G., Mitsuyasu, R. T., Symonds, G., Rosenblatt, J. D., Zack, J., Sun, L. Q., Miller, M., Ely, J., and Gerlach, W. (1999). A phase I trial of autologous CD34+ hematopoietic progenitor cells transduced with an anti-HIV ribozyme. *Hum. Gene Ther.* **10**, 2255–2270.

Amado, R. G., Mitsuyasu, R. T., Rosenblatt, J. D., Ngok, F. K., Bakker, A., Cole, S., Chorn, N., Lin, L. S., Bristol, G., Boyd, M. P., MacPherson, J. L., Fanning, G. C., *et al.* (2004).

Anti-human immunodeficiency virus hematopoietic progenitor cell-delivered ribozyme in a phase I study: Myeloid and lymphoid reconstitution in human immunodeficiency virus type-1-infected patients. *Hum. Gene Ther.* **15**, 251–262.

An, D. S., Qin, F. X., Auyeung, V. C., Mao, S. H., Kung, S. K., Baltimore, D., and Chen, I. S. (2006). Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol. Ther.* **14**, 494–504.

Anderson, J., and Akkina, R. (2005). HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. *AIDS Res. Ther.* **2**, 1.

Arteaga, H. J., Hinkula, J., van Dijk-Hard, I., Dilber, M. S., Wahren, B., Christensson, B., Mohamed, A. J., and Smith, C. I. (2003). Choosing CCR5 or Rev siRNA in HIV-1. *Nat. Biotechnol.* **21**, 230–231.

Bahner, I., Sumiyoshi, T., Kagoda, M., Swartout, R., Peterson, D., Pepper, K., Dorey, F., Reiser, J., and Kohn, D. B. (2007). Lentiviral vector transduction of a dominant-negative *Rev* gene into human CD34(+) hematopoietic progenitor cells potently inhibits human immunodeficiency virus-1 replication. *Mol. Ther.* **15**, 76–85.

Bai, J., Banda, N., Lee, N. S., Rossi, J., and Akkina, R. (2002). RNA-based anti-HIV-1 gene therapeutic constructs in SCID-hu mouse model. *Mol. Ther.* **6**, 770–782.

Bai, J., Sui, J., Zhu, R. Y., Tallarico, A. S., Gennari, F., Zhang, D., and Marasco, W. A. (2003). Inhibition of Tat-mediated transactivation and HIV-1 replication by human anti-hCyclinT1 intrabodies. *J. Biol. Chem.* **278**, 1433–1442.

Baltimore, D. (1988). Gene therapy. Intracellular immunization. *Nature* **335**, 395–396.

Bauer, G., Valdez, P., Kearns, K., Bahner, I., Wen, S. F., Zaia, J. A., and Kohn, D. B. (1997). Inhibition of human immunodeficiency virus-1 (HIV-1) replication after transduction of granulocyte colony-stimulating factor-mobilized CD34 + cells from HIV-1-infected donors using retroviral vectors containing anti-HIV-1 genes. *Blood* **89**, 2259–2267.

Bennasser, Y., Le, S. Y., Yeung, M. L., and Jeang, K. T. (2004). HIV-1 encoded candidate micro-RNAs and their cellular targets. *Retrovirology* **1**, 43.

Bennasser, Y., Le, S. Y., Benkirane, M., and Jeang, K. T. (2005). Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity* **22**, 607–619.

Bevec, D., Dobrovnik, M., Hauber, J., and Bohnlein, E. (1992). Inhibition of human immunodeficiency virus type 1 replication in human T cells by retroviral-mediated gene transfer of a dominant-negative Rev trans- activator. *Proc. Natl. Acad. Sci. USA* **89**, 9870–9874.

Boden, D., Pusch, O., Lee, F., Tucker, L., and Ramratnam, B. (2003). Human immunodeficiency virus type 1 escape from RNA interference. *J. Virol.* **77**, 11531–11535.

Bridges, S. H., and Sarver, N. (1995). Gene therapy and immune restoration for HIV disease. *Lancet* **345**, 427–432.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., *et al.* (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**, 669–672.

Chan, S. Y., Louie, M. C., Piccotti, J. R., Iyer, G., Ling, X., Yang, Z. Y., Nabel, G. J., and Bishop, D. K. (1998). Genetic vaccination-induced immune responses to the human immunodeficiency virus protein Rev: Emergence of the interleukin 2-producing helper T lymphocyte. *Hum. Gene Ther.* **9**, 2187–2196.

Chang, A. H., and Sadelain, M. (2007). The genetic engineering of hematopoietic stem cells: The rise of lentiviral vectors, the conundrum of the LTR, and the promise of lineage-restricted vectors. *Mol. Ther.* **15**, 445–456.

Chen, X., Barfield, R., Benaim, E., Leung, W., Knowles, J., Lawrence, D., Otto, M., Shurtleff, S. A., Neale, G. A., Behm, F. G., Turner, V., and Handgretinger, R. (2005). Prediction of T-cell reconstitution by assessment of T-cell receptor excision circle before allogeneic hematopoietic stem cell transplantation in pediatric patients. *Blood* **105**, 886–893.

Chun, T. W., and Fauci, A. S. (1999). Latent reservoirs of HIV: Obstacles to the eradication of virus. *Proc. Natl. Acad. Sci. USA* **96**, 10958–10961.

Chun, T. W., Davey, R. T., Jr., Engel, D., Lane, H. C., and Fauci, A. S. (1999). Re-emergence of HIV after stopping therapy. *Nature* **401**, 874–875.

Clotet, B. (2004). Strategies for overcoming resistance in HIV-1 infected patients receiving HAART. *AIDS Rev.* **6**, 123–130.

Cullen, B. R. (2005). Does RNA interference have a future as a treatment for HIV-1 induced disease? *AIDS Rev.* **7**, 22–25.

Das, A. T., Brummelkamp, T. R., Westerhout, E. M., Vink, M., Madiredjo, M., Bernards, R., and Berkhout, B. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.* **78**, 2601–2605.

Dave, U. P., Jenkins, N. A., and Copeland, N. G. (2004). Gene therapy insertional mutagenesis insights. *Science* **303**, 333.

Davis, B. M., Reese, J. S., Koc, O. N., Lee, K., Schupp, J. E., and Gerson, S. L. (1997). Selection for G156A O6-methylguanine DNA methyltransferase gene-transduced hematopoietic progenitors and protection from lethality in mice treated with O6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.* **57**, 5093–5099.

Davis, B. M., Humeau, L., and Dropulic, B. (2004). *In vivo* selection for human and murine hematopoietic cells transduced with a therapeutic MGMT lentiviral vector that inhibits HIV replication. *Mol. Ther.* **9**, 160–172.

Donahue, R. E., Bunnell, B. A., Zink, M. C., Metzger, M. E., Westro, R. P., Kirby, M. R., Unangst, T., Clements, J. E., and Morgan, R. A. (1998). Reduction in SIV replication in rhesus macaques infused with autologous lymphocytes engineered with antiviral genes. *Nat. Med.* **4**, 181–186.

Dore, G. J., and Cooper, D. A. (2006). HAART's first decade: Success brings further challenges. *Lancet* **368**, 427–428.

Douek, D. C., McFarland, R. D., Keiser, P. H., Gage, E. A., Massey, J. M., Haynes, B. F., Polis, M. A., Haase, A. T., Feinberg, M. B., Sullivan, J. L., Jamieson, B. D., Zack, J. A., *et al.* (1998). Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690–695.

Eckert, D. M., and Kim, P. S. (2001). Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* **70**, 777–810.

Egelhofer, M., Brandenburg, G., Martinius, H., Schult-Dietrich, P., Melikyan, G., Kunert, R., Baum, C., Choi, I., Alexandrov, A., and von Laer, D. (2004). Inhibition of human immunodeficiency virus type 1 entry in cells expressing gp41-derived peptides. *J. Virol.* **78**, 568–575.

Fassati, A. (2006). HIV infection of non-dividing cells: A divisive problem. *Retrovirology* **3**, 74.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.

Gabarre, J., Azar, N., Autran, B., Katlama, C., and Leblond, V. (2000). High-dose therapy and autologous haematopoietic stem-cell transplantation for HIV-1-associated lymphoma. *Lancet* **355**, 1071–1072.

Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* **107**, 55–65.

Goila, R., and Banerjea, A. C. (1998). Sequence specific cleavage of the HIV-1 coreceptor CCR5 gene by a hammer-head ribozyme and a DNA-enzyme: Inhibition of the coreceptor function by DNA-enzyme. *FEBS Lett.* **436**, 233–238.

Gonzalez, M. A., Serrano, F., Llorente, M., Abad, J. L., Garcia-Ortiz, M. J., and Bernad, A. (1998). A hammerhead ribozyme targeted to the human chemokine receptor CCR5. *Biochem. Biophys. Res. Commun.* **251**, 592–596.

Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F., and Kay, M. A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537–541.

Guillaume, T., Rubinstein, D. B., and Symann, M. (1998). Immune reconstitution and immunotherapy after autologous hematopoietic stem cell transplantation. *Blood* **92**, 1471–1490.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J. L., Fraser, C. C., Cavazzana-Calvo, M., and Fischer, A. (2003a). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **348**, 255–256.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., *et al.* (2003b). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419.

Haseloff, J., and Gerlach, W. L. (1988). Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* **334**, 585–591.

Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K., Erickson, D., *et al.* (1996). The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat. Med.* **2**, 1240–1243.

Jacque, J. M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438.

Jamieson, B. D., Douek, D. C., Killian, S., Hultin, L. E., Scripture-Adams, D. D., Giorgi, J. V., Marelli, D., Koup, R. A., and Zack, J. A. (1999). Generation of functional thymocytes in the human adult. *Immunity* **10**, 569–575.

Kang, E. M., de Witte, M., Malech, H., Morgan, R. A., Phang, S., Carter, C., Leitman, S. F., Childs, R., Barrett, A. J., Little, R., and Tisdale, J. F. (2002). Nonmyeloablative conditioning followed by transplantation of genetically modified HLA-matched peripheral blood progenitor cells for hematologic malignancies in patients with acquired immunodeficiency syndrome. *Blood* **99**, 698–701.

Kohn, D. B., Bauer, G., Rice, C. R., Rothschild, J. C., Carbonaro, D. A., Valdez, P., Hao, Q., Zhou, C., Bahner, I., Kearns, K., Brody, K., Fox, S., *et al.* (1999). A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood* **94**, 368–371.

Kohn, D. B., Sadelain, M., Dunbar, C., Bodine, D., Kiem, H. P., Candotti, F., Tisdale, J., Riviere, I., Blau, C. A., Richard, R. E., Sorrentino, B., Nolta, J., *et al.* (2003). American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. *Mol. Ther.* **8**, 180–187.

Krishnan, A., Zaia, J., and Molina, A. (2002). Stem cell transplantation and gene therapy for HIV-related lymphomas. *J. Hematother. Stem Cell Res.* **11**, 765–775.

Krishnan, A., Molina, A., Zaia, J., Smith, D., Vasquez, D., Kogut, N., Falk, P. M., Rosenthal, J., Alvarnas, J., and Forman, S. J. (2005). Durable remissions with autologous stem cell transplantation for high-risk HIV-associated lymphomas. *Blood* **105**, 874–878.

Kustikova, O., Fehse, B., Modlich, U., Yang, M., Dullmann, J., Kamino, K., von Neuhoff, N., Schlegelberger, B., Li, Z., and Baum, C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. *Science* **308**, 1171–1174.

Lalezari, J. P., Henry, K., O'Hearn, M., Montaner, J. S., Piliero, P. J., Trottier, B., Walmsley, S., Cohen, C., Kuritzkes, D. R., Eron, J. J., Jr., Chung, J., DeMasi, R., *et al.* (2003). Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. *N. Engl. J. Med.* **348**, 2175–2185.

Laufs, S., Gentner, B., Nagy, K. Z., Jauch, A., Benner, A., Naundorf, S., Kuehlcke, K., Schiedlmeier, B., Ho, A. D., Zeller, W. J., and Fruehauf, S. (2003). Retroviral vector

integration occurs in preferred genomic targets of human bone marrow-repopulating cells. *Blood* **101**, 2191–2198.

Lee, M. T., Coburn, G. A., McClure, M. O., and Cullen, B. R. (2003). Inhibition of human immunodeficiency virus type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector. *J. Virol.* **77**, 11964–11972.

Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.* **20**, 500–505.

Levine, B. L., Humeau, L. M., Boyer, J., MacGregor, R. R., Rebello, T., Lu, X., Binder, G. K., Slepushkin, V., Lemiale, F., Mascola, J. R., Bushman, F. D., Dropulic, B., *et al.* (2006). Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc. Natl. Acad. Sci. USA* **103**, 17372–17377.

Li, M. J., Bauer, G., Michienzi, A., Yee, J. K., Lee, N. S., Kim, J., Li, S., Castanotto, D., Zaia, J., and Rossi, J. J. (2003). Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol. Ther.* **8**, 196–206.

Li, M. J., Kim, J., Li, S., Zaia, J., Yee, J. K., Anderson, J., Akkina, R., and Rossi, J. J. (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol. Ther.* **12**, 900–909.

Lim, J. K., Glass, W. G., McDermott, D. H., and Murphy, P. M. (2006). CCR5: No longer a "good for nothing" gene—chemokine control of West Nile virus infection. *Trends Immunol.* **27**, 308–312.

Liu, D., Donegan, J., Nuovo, G., Mitra, D., and Laurence, J. (1997). Stable human immunodeficiency virus type 1 (HIV-1) resistance in transformed $CD4^+$ monocytic cells treated with multitargeting HIV-1 antisense sequences incorporated into U1 snRNA. *J. Virol.* **71**, 4079–4085.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367–377.

Lobato, M. N., and Rabbitts, T. H. (2003). Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol. Med.* **9**, 390–396.

Looney, D. J., Poeschla, E., Leavitt, M. C., Gilles, P., Marich, J., Robbins, J., Haidri, P., Barber, J., Yu, M., and Wong-Staal, F. (1998). T-cell ribozyme gene therapy of HIV-1 infection: Progress report from a phase I trial. *5th Conference on Retroviruses and Opportunistic Infections*, Chicago.

Luis Abad, J., Gonzalez, M. A., del Real, G., Mira, E., Manes, S., Serrano, F., and Bernad, A. (2003). Novel interfering bifunctional molecules against the CCR5 coreceptor are efficient inhibitors of HIV-1 infection. *Mol. Ther.* **8**, 475–484.

MacGregor, R. R. (2001). Clinical protocol. A phase 1 open-label clinical trial of the safety and tolerability of single escalating doses of autologous CD4 T cells transduced with VRX496 in HIV-positive subjects. *Hum. Gene Ther.* **12**, 2028–2029.

Macpherson, J. L., Ely, J. A., Sun, L. Q., and Symonds, G. P. (1999). Ribozymes in gene therapy of HIV-1. *Front. Biosci.* **4**, D497–D505.

Macpherson, J. L., Boyd, M. P., Arndt, A. J., Todd, A. V., Fanning, G. C., Ely, J. A., Elliott, F., Knop, A., Raponi, M., Murray, J., Gerlach, W., Sun, L. Q., *et al.* (2005). Long-term survival and concomitant gene expression of ribozyme-transduced $CD4^+$ T-lymphocytes in HIV-infected patients. *J. Gene Med.* **7**, 552–564.

Malim, M. H., Bohnlein, S., Hauber, J., and Cullen, B. R. (1989). Functional dissection of the HIV-1 Rev trans-activator—derivation of a trans-dominant repressor of Rev function. *Cell* **58**, 205–214.

Malim, M. H., Freimuth, W. W., Liu, J., Boyle, T. J., Lyerly, H. K., Cullen, B. R., and Nabel, G. J. (1992). Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. *J. Exp. Med.* **176**, 1197–1201.

Manilla, P., Rebello, T., Afable, C., Lu, X., Slepushkin, V., Humeau, L. M., Schonely, K., Ni, Y., Binder, G. K., Levine, B. L., MacGregor, R. R., June, C. H., *et al.* (2005). Regulatory considerations for novel gene therapy products: A review of the process leading to the first clinical lentiviral vector. *Hum. Gene Ther.* **16**, 17–25.

Marcello, A., Lusic, M., Pegoraro, G., Pellegrini, V., Beltram, F., and Giacca, M. (2004). Nuclear organization and the control of HIV-1 transcription. *Gene* **326**, 1–11.

Martinez, M. A., Gutierrez, A., Armand-Ugon, M., Blanco, J., Parera, M., Gomez, J., Clotet, B., and Este, J. A. (2002). Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. *AIDS* **16**, 2385–2390.

Mautino, M. R., and Morgan, R. A. (2002). Inhibition of HIV-1 replication by novel lentiviral vectors expressing transdominant Rev and HIV-1 env antisense. *Gene Ther.* **9**, 421–431.

May, M. T., Sterne, J. A., Costagliola, D., Sabin, C. A., Phillips, A. N., Justice, A. C., Dabis, F., Gill, J., Lundgren, J., Hogg, R. S., de Wolf, F., Fatkenheuer, G., *et al.* (2006). HIV treatment response and prognosis in Europe and North America in the first decade of highly active antiretroviral therapy: A collaborative analysis. *Lancet* **368**, 451–458.

Michienzi, A., Castanotto, D., Lee, N., Li, S., Zaia, J. A., and Rossi, J. J. (2003). RNA-mediated inhibition of HIV in a gene therapy setting. *Ann. N. Y. Acad. Sci.* **1002**, 63–71.

Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999). Transduction of human CD34 + cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283**, 682–686.

Morgan, R. A., and Walker, R. (1996). Gene therapy for AIDS using retroviral mediated gene transfer to deliver HIV-1 antisense TAR and transdominant Rev protein genes to syngeneic lymphocytes in HIV-1 infected identical twins. *Hum. Gene Ther.* **7**, 1281–1306.

Morgan, R. A., Walker, R., Carter, C. S., Natarajan, V., Tavel, J. A., Bechtel, C., Herpin, B., Muul, L., Zheng, Z., Jagannatha, S., Bunnell, B. A., Fellowes, V., *et al.* (2005). Preferential survival of $CD4^+$ T lymphocytes engineered with anti-human immunodeficiency virus (HIV) genes in HIV-infected individuals. *Hum. Gene Ther.* **16**, 1065–1074.

Morris, K. V., and Rossi, J. J. (2006). Lentiviral-mediated delivery of siRNAs for antiviral therapy. *Gene Ther.* **13**, 553–558.

Muul, L. M., Tuschong, L. M., Soenen, S. L., Jagadeesh, G. J., Ramsey, W. J., Long, Z., Carter, C. S., Garabedian, E. K., Alleyne, M., Brown, M., Bernstein, W., Schurman, S. H., *et al.* (2003). Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: Long-term results of the first clinical gene therapy trial. *Blood* **101**, 2563–2569.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D. (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267.

Nienhuis, A. W., Dunbar, C. E., and Sorrentino, B. P. (2006). Genotoxicity of retroviral integration in hematopoietic cells. *Mol. Ther.* **13**, 1031–1049.

Nobile, M., Correa, R., Borghans, J. A., D'Agostino, C., Schneider, P., De Boer, R. J., and Pantaleo, G. (2004). De novo T-cell generation in patients at different ages and stages of HIV-1 disease. *Blood* **104**, 470–477.

Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P. J., Riess, J., Lee, S. K., Collman, R. G., Lieberman, J., Shankar, P., and Sharp, P. A. (2002). siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* **8**, 681–686.

Omoto, S., and Fujii, Y. R. (2005). Regulation of human immunodeficiency virus 1 transcription by nef microRNA. *J. Gen. Virol.* **86**, 751–755.

Ott, M. G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kuhlcke, K., Schilz, A., Kunkel, H., Naundorf, S., Brinkmann, A., *et al.* (2006).

Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat. Med.* **12**, 401–409.

Palella, F. J., Jr., Delaney, K. M., Moorman, A. C., Loveless, M. O., Fuhrer, J., Satten, G. A., Aschman, D. J., and Holmberg, S. D. (1998). Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N. Engl. J. Med.* **338**, 853–860.

Persaud, D., Zhou, Y., Siliciano, J. M., and Siliciano, R. F. (2003). Latency in human immunodeficiency virus type 1 infection: No easy answers. *J. Virol.* **77**, 1659–1665.

Podsakoff, G. M., Engel, B. C., Carbonaro, D. A., Choi, C., Smogorzewska, E. M., Bauer, G., Selander, D., Csik, S., Wilson, K., Betts, M. R., Koup, R. A., Nabel, G. J., *et al.* (2005). Selective survival of peripheral blood lymphocytes in children with HIV-1 following delivery of an anti-HIV gene to bone marrow CD34(+) cells. *Mol. Ther.* **12**, 77–86.

Poulin, J. F., Viswanathan, M. N., Harris, J. M., Komanduri, K. V., Wieder, E., Ringuette, N., Jenkins, M., McCune, J. M., and Sekaly, R. P. (1999). Direct evidence for thymic function in adult humans. *J. Exp. Med.* **190**, 479–486.

Proudfoot, A. E. (2002). Chemokine receptors: Multifaceted therapeutic targets. *Nat. Rev. Immunol.* **2**, 106–115.

Provost, P., Barat, C., Plante, I., and Tremblay, M. J. (2006). HIV-l and the microRNA-guided silencing pathway: An intricate and multifaceted encounter. *Virus Res.* **121**, 107–115.

Qin, X. F., An, D. S., Chen, I. S., and Baltimore, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. USA* **100**, 183–188.

Qureshi, A., Zheng, R., Parlett, T., Shi, X., Balaraman, P., Cheloufi, S., Murphy, B., Guntermann, C., and Eagles, P. (2006). Gene silencing of HIV chemokine receptors using ribozymes and single-stranded antisense RNA. *Biochem. J.* **394**, 511–518.

Ranga, U., Woffendin, C., Verma, S., Xu, L., June, C. H., Bishop, D. K., and Nabel, G. J. (1998). Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. *Proc. Natl. Acad. Sci. USA* **95**, 1201–1206.

Rondon, I. J., and Marasco, W. A. (1997). Intracellular antibodies (intrabodies) for gene therapy of infectious diseases. *Annu. Rev. Microbiol.* **51**, 257–283.

Rossi, J. J. (1999). The application of ribozymes to HIV infection. *Curr. Opin. Mol. Ther.* **1**, 316–322.

Rossi, J. J. (2006). RNAi as a treatment for HIV-1 infection. *Biotechniques* (Suppl), 25–29.

Sabariegos, R., Gimenez-Barcons, M., Tapia, N., Clotet, B., and Martinez, M. A. (2006). Sequence homology required by human immunodeficiency virus type 1 to escape from short interfering RNAs. *J. Virol.* **80**, 571–577.

Sakuma, R., Noser, J. A., Ohmine, S., and Ikeda, Y. (2007). Inhibition of HIV-1 replication by simian restriction factors, TRIM5alpha and APOBEC3G. *Gene Ther.* **14**, 185–189.

Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., L apoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., *et al.* (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722–725.

Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A., and Rossi, J. J. (1990). Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**, 1222–1225.

Schambach, A., Schiedlmeier, B., Kuhlcke, K., Verstegen, M., Margison, G. P., Li, Z., Kamino, K., Bohne, J., Alexandrov, A., Hermann, F. G., von Laer, D., and Baum, C. (2006). Towards hematopoietic stem cell-mediated protection against infection with human immunodeficiency virus. *Gene Ther.* **13**, 1037–1047.

Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R., and Bushman, F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**, 521–529.

Sharp, P. A. (2001). RNA interference—2001. *Genes Dev.* **15**, 485–490.

Siliciano, J. D., Kajdas, J., Finzi, D., Quinn, T. C., Chadwick, K., Margolick, J. B., Kovacs, C., Gange, S. J., and Siliciano, R. F. (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. *Nat. Med.* **9**, 727–728.

Steinberger, P., Andris-Widhopf, J., Buhler, B., Torbett, B. E., and Barbas, C. F., 3rd. (2000). Functional deletion of the CCR5 receptor by intracellular immunization produces cells that are refractory to CCR5-dependent HIV-1 infection and cell fusion. *Proc. Natl. Acad. Sci. USA* **97**, 805–810.

Strayer, D. S., Akkina, R., Bunnell, B. A., Dropulic, B., Planelles, V., Pomerantz, R. J., Rossi, J. J., and Zaia, J. A. (2005). Current status of gene therapy strategies to treat HIV/AIDS. *Mol. Ther.* **11**, 823–842.

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427**, 848–853.

Sun, L. Q., Wang, L., Gerlach, W. L., and Symonds, G. (1995). Target sequence-specific inhibition of HIV-1 replication by ribozymes directed to tat RNA. *Nucleic Acids Res.* **23**, 2909–2913.

Sutton, R. E., Wu, H. T., Rigg, R., Bohnlein, E., and Brown, P. O. (1998). Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J. Virol.* **72**, 5781–5788.

Talvensaari, K., Clave, E., Douay, C., Rabian, C., Garderet, L., Busson, M., Garnier, F., Douek, D., Gluckman, E., Charron, D., and Toubert, A. (2002). A broad T-cell repertoire diversity and an efficient thymic function indicate a favorable long-term immune reconstitution after cord blood stem cell transplantation. *Blood* **99**, 1458–1464.

ter Brake, O., Konstantinova, P., Ceylan, M., and Berkhout, B. (2006). Silencing of HIV-1 with RNA interference: A multiple shRNA approach. *Mol. Ther.* **14**, 883–892.

Thrasher, A. J., Gaspar, H. B., Baum, C., Modlich, U., Schambach, A., Candotti, F., Otsu, M., Sorrentino, B., Scobie, L., Cameron, E., Blyth, K., Neil, J., *et al.* (2006). Gene therapy: X-SCID transgene leukaemogenicity. *Nature* **443**, E5–E6; discussion E6–E7.

Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., Lebrigand, K., Cardinaud, B., Maurin, T., Barbry, P., Baillat, V., Reynes, J., Corbeau, P., *et al.* (2007). Suppression of MicroRNA-Silencing Pathway by HIV-1 During Virus Replication. *Science* **315**, 1579–1582.

Tuschl, T. (2002). Expanding small RNA interference. *Nat. Biotechnol.* **20**, 446–448.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M., and Kappes, J. C. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* **46**, 1896–1905.

Weinberg, K., Blazar, B. R., Wagner, J. E., Agura, E., Hill, B. J., Smogorzewska, M., Koup, R. A., Betts, M. R., Collins, R. H., and Douek, D. C. (2001). Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood* **97**, 1458–1466.

Westerhout, E. M., Ooms, M., Vink, M., Das, A. T., and Berkhout, B. (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.* **33**, 796–804.

Westerhout, E. M., ter Brake, O., and Berkhout, B. (2006). The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference. *Retrovirology* **3**, 57.

Woffendin, C., Ranga, U., Yang, Z., Xu, L., and Nabel, G. J. (1996). Expression of a protective gene-prolongs survival of T cells in human immunodeficiency virus-infected patients. *Proc. Natl. Acad. Sci. USA* **93**, 2889–2894.

Wolkowicz, R., and Nolan, G. P. (2005). Gene therapy progress and prospects: Novel gene therapy approaches for AIDS. *Gene Ther.* **12**, 467–476.

Wong-Staal, F., Poeschla, E. M., and Looney, D. J. (1998). A controlled, Phase 1 clinical trial to evaluate the safety and effects in HIV-1 infected humans of autologous lymphocytes transduced with a ribozyme that cleaves HIV-1 RNA. *Hum. Gene Ther.* **9**, 2407–2425.

Woods, N. B., Bottero, V., Schmidt, M., von Kalle, C., and Verma, I. M. (2006). Gene therapy: Therapeutic gene causing lymphoma. *Nature* **440**, 1123.

Wu, X., Li, Y., Crise, B., and Burgess, S. M. (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**, 1749–1751.

Yang, X., Gold, M. O., Tang, D. N., Lewis, D. E., Aguilar-Cordova, E., Rice, A. P., and Herrmann, C. H. (1997). TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. *Proc. Natl. Acad. Sci. USA* **94**, 12331–12336.

Yu, M., Poeschla, E., and Wong-Staal, F. (1994). Progress towards gene therapy for HIV infection. *Gene Ther.* **1**, 13–26.

Zaia, J. A. (2003). Problems and solutions to successful gene-transfer based therapies for HIV. *Clin. Appl. Immunol. Rev.* **3**, 199–211.

This page intentionally left blank

Zachary A. Klase, Rachel Van Duyne, and Fatah Kashanchi

Department of Biochemistry, Medical Center, The George Washington University, Washington, DC 20037

Identification of Potential Drug Targets Using Genomics and Proteomics: A Systems Approach

I. Chapter Overview

Current *human immunodeficiency virus 1* (HIV-1) antiviral therapies have proven to be insufficient and limited due to the ability of the virus to develop resistant mutants. Specific cellular targets are needed for the next generation of HIV-1 therapeutics. Emerging genomic and proteomic techniques have elucidated a myriad of cellular genes involved in viral replication representing a collection of possible new drug targets. Here, the cellular–viral interactions of viral proteins are introduced and expanded

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56011-4

upon to elucidate phenotypic changes as well as variations in cellular gene expression levels induced by HIV-1 infection. The collective organization of data utilizing a systems approach allows for the mapping of complex pathways and interactions between otherwise unknown protein partners. In support of this approach, we examine the multitude of cellular changes related to the expression of Tat, Nef, and Gag. In closing, we demonstrate how counteracting specific effects of viral proteins can alter disease pathogenesis.

II. Introduction

The race to complete the human genome as well as the successful sequencing of the genomes of many other organisms has provided modern science with an array of tools that promise to facilitate advancements in many branches of biological research. Genomics is classified as the study of an organism's genome through the expression and function of its genes. This field of study is concerned with the systematic use of genome information, associated with other data and is made possible by a series of techniques related to identification of genes and the level of gene expression. Foremost among genomic techniques is the oligonucleotide microarray, which when applied to cDNA derived from RNA of a sample population allows for the assessment of the total level of mRNA transcripts in a cell. These levels are influenced by the rates of transcription and turnover of each mRNA sequence and are often indicative of changes in protein expression. When used comparatively, the oligonucleotide microarray allows changes in mRNA levels between samples, such as normal versus diseased, to be monitored in cells or tissues. Additionally, array technology has been used to identify polymorphisms in gene coding sequences, vouching for the specificity and sensitivity of this genomic approach. Adaptation of this technology to viral sequences allows for the monitoring of viral RNA levels with relative ease.

Following the central dogma of molecular biology, the field of genomics has progressed to its protein equivalent, proteomics. Proteomics is defined as the large-scale study of proteins and their interactions through structure and function, therefore defining an organism's proteome. The mechanism behind defining a proteome follows two basic steps: first, the separation of proteins from a complex protein mixture, followed by the identification/characterization of the single protein or complex based on comparison to a defined protein sequence database. These two aspects of protein identification are often performed through the utilization of front-end purification techniques such as liquid chromatography (LC) or 2D gel electrophoresis (2DGE) followed by mass spectrometry (MS). LC can be directly coupled to a mass spectrometer such that a complex protein mixture is infused into a high performance liquid chromatography (HPLC) system, separated based

on size, charge, or affinity into fractions that are directly injected into an MS system therefore reducing the complexity of the samples and allowing for the direct identification of the protein(s) of interest. Additionally, a 2DGE approach for front-end purification results in a visual separation of a mixture of proteins in two dimensions based on isoelectric point and mass. A comparative analysis of protein spots from 2D gels representing different cellular states provides insight into changes in protein expression levels. These protein spots can be excised from the 2D gel and submitted to an MS system for analysis. Proteins may be isolated based on tissue type, cell type, and organelle localization in order to monitor changes in protein levels at specific locations, which can give clues to alterations such as protein trafficking, expression, and localization. These standard proteomic techniques are adaptable to subdivisions of the proteome such as the interactome, glycome, metabalome.

The fusion of genomics and proteomics has led to an array of techniques for studying the overall state of RNA transcription and protein expression in a cell. Chromatin immunoprecipitation (ChIP), for example, allows DNA associated with a given protein to be captured and analyzed by polymerase chain reaction, sequencing of recovered DNA, or microarray. Initial screening by a genomic or proteomic technique may identify patterns that can be followed up by more directed analysis with another technique. Overall, the disciplines of genomics and proteomics allow for the rapid identification of the overall cellular environment and can be applied to identify proteins and processes involved in HIV-1 pathogenesis and replication as well as the identification of novel pathways. Many studies have been performed on HIV-1 using these genomic and proteomic approaches. In this chapter, we will discuss some of the work published in the field while providing examples of how these approaches may be integrated to yield a greater understanding of the way in which HIV-1 influences the host cell. Examination of these studies will assess the current state of genomics and proteomics as applied to the field of human retrovirology as well as to identify key points of interest for future studies.

III. Viral Targets

A. Viral Genomics

The HIV-1 genome includes nine viral genes. All the viral genes are expressed from a single promoter located in the viral long-terminal repeat (LTR) (Coffin *et al.*, 1997; Greene and Peterlin, 2002); three of these genes (*Gag*, *Pol*, and *Env*) are common to all lentiviruses and the other six (*Tat*, *Rev*, *Nef*, *Vpr*, *Vpu*, and *Vif*) are referred to as accessory genes. *Tat*, the viral transactivator, feeds back on the viral promoter and increases transcription (Bohan *et al.*, 1992; Feinberg *et al.*, 1991; Jeang *et al.*,

1999; Laspia *et al.*, 1989). Temporal regulation of protein expression is achieved through alternative splicing regulated by the viral protein Rev (Cullen, 1991; Fukumori *et al.*, 1999; Hope, 1997; Hope and Pomerantz, 1995). Rev serves to stabilize transcripts and leads to the production of singly spliced and finally unspliced messages. In this way, viral gene expression is temporally regulated such that each protein is produced as needed (Coffin *et al.*, 1997; Fukumori *et al.*, 1999; Greene and Peterlin, 2002).

Although the regulation of viral gene expression was elucidated before the advent of genomics, modern techniques can still be useful in studying viral gene expression. Recently, the development of viral gene arrays allows for the detection of genomic DNA or viral transcripts. These arrays are capable of detecting sequence from all the open reading frames of HIV-1, human T-cell leukemia virus types 1 and 2, *Hepatitis C virus*, Epstein-Barr virus, human herpesvirus 6A and 6B, poxviruses, Varicella-zoster virus, and Kaposi's sarcoma-associated herpesvirus (Cohrs *et al.*, 2006; Conejero-Goldberg *et al.*, 2005; Ghedin *et al.*, 2004; Kostrzynska and Bachand, 2006; Ryabinin *et al.*, 2006; Yuan *et al.*, 2006). Initial experiments show its utility in detecting expression of viral genes and in identifying proteins bound to the viral genome through ChIP–chip analysis (Ghedin *et al.*, 2004). Use of a viral chip will provide researchers with a new tool for diagnostics, examination of viral gene expression under different conditions, and the identification of the interplay between viruses in coinfection. Indeed, the viral array has been used to examine the effect of the CDK inhibitor Cyc202 on viral gene expression (Ghedin *et al.*, 2004). As pharmaceuticals targeting various stages of the viral life cycle within the infected cell become available, technologies such as the viral array will prove invaluable in examining their effects. Future work in this field will likely focus on examining changes in the expression level of specific viral genes in specific cell types and in response to therapies.

B. Current Therapies and Future Prospects

When administered properly, the currently available HIV medications are capable of controlling infection, they do not, however, represent a cure. While the drugs merely prevent infection of new cells, latently infected cells continue to produce varying levels of wild-type and mutant virus. If therapy is discontinued, long-lived reservoirs of infected cells are capable of producing infectious virus and continuing the progression to AIDS. Additionally, the currently available drugs specifically target viral proteins such that small polymorphisms largely affect the efficiency of drug action. The high rate of mutation caused by the viral reverse transcriptase enzyme, for example, and the large numbers of new virus produced during each round of infection allow the virus to select variants with mutations that make them resistant to currently available therapies.

Current therapies in HIV are insufficient due to their inability to cure the disease and the ability of the virus to become resistant to the treatment over time. New therapies must be developed that target new mechanisms important to the viral life cycle. HIV encodes only nine genes; a fact that forces the virus to interact with and subvert cellular processes for its own benefit. Identifying these interactions and their effect on both the virus and the cell will reveal a new range of targets for future therapeutics.

The use of broad and powerful approaches afforded to us by the omics age will enable identification of such targets. First and foremost to this approach is the understanding of the contact point between the virus and the cell—that is, the interaction between viral components and cellular components. It is this interaction that allows the virus to influence cellular processes. Second, we must understand the effect this interaction has on the system as a whole. Finally, we can map this interaction to specific pathways within the cell, thereby identifying a mechanism for the observed effect. Once a target has been discovered, a drug can be selected that counteracts this effect by either preventing the initial interaction or altering the affected pathway to counteract this interaction.

In this chapter, we will discuss the important aspects of understanding the virus effect on the host organism using a systems approach. A sampling of the many interactions that HIV has with its host will be incorporated with a discussion of their overall effect, followed by a look into the identification of potential drug targets. Tat, Nef, and Gag will be used as examples of how to follow the effect of one viral protein through the cellular system to a potential drug target. Instead of providing a broad picture of how modern proteomic and genomic approaches can be used to identify potential drug targets, we will provide specific examples throughout the chapter.

IV. Cellular/Viral Protein–Protein Interactions

The study of protein–protein interactions between HIV-1 and the host cell provides an important insight in the mechanisms that allow the virus to manipulate cellular activities and/or alter cell regulatory mechanisms for its benefit (Tasara *et al.*, 2001). In order for HIV-1 to efficiently replicate in a susceptible host, cellular proteins must first be incorporated into the virion. Throughout the viral life cycle, events such as the assembly of the preintegration complex (PIC), reverse transcription of the HIV-1 genome, viral gene expression, viral assembly, and budding are all the results of specific viral–host protein interactions (Bannwarth and Gatignol, 2005; Bieniasz *et al.*, 1999; Bryant and Ratner, 1990; Ciborowski and Gendelman, 2006; Freed, 2002; Hope, 1997; Misumi *et al.*, 2002).

Identification of cofactors involved in HIV-1 infection and/or replication can be accomplished based on the molecular interactions between viral

and cellular proteins (Tang, 2002). To date, interactions between viral and host cellular proteins have been identified and characterized using classical scientific approaches appropriate to the field of virology (Kellam, 2001). As the HIV-1 genome consists of only nine viral genes, the characterization of the interactome, the complete set of protein–protein interactions, is a tangible goal. Prior to the advent of the "-omics" world, viral–host interactions were investigated solely using established experimental biology techniques and research methods that should now complement postgenomic methods rather than being replaced (Kellam, 2001). A collection of HIV-1–host protein interactions are displayed in Table I; though not exhaustive, the list of interactions establishes the major proteins responsible for successful viral infection, replication, and immune suppression, among others.

Fortunately, virology has caught up to emerging sciences and as a result has incorporated genomic and proteomic techniques into the study of infectivity and therapeutics. The utilization of high throughput postgenomic research techniques such as gene expression microarrays and protein arrays, LC, yeast two-hybrid (Y2H) screens, and MS allows for the rapid detection of viral–host protein interactions and aids in the understanding and manipulation of the associated viral and cellular pathways (Kellam, 2001).

A. Cellular Protein Interactions of Tat

Tat has been known to not only stimulate the HIV LTR promoter but also modulate and induce cellular genes. Historically, the mechanism of action by Tat has been assigned to the level of initiation and elongation (Bohan *et al.*, 1992; Feinberg *et al.*, 1991; Kato *et al.*, 1992; Laspia *et al.*, 1989; Marciniak and Sharp, 1991; Marciniak *et al.*, 1990). The effect of Tat on preinitiation, initiation, and elongation has been observed through a number of biochemical interactions including physical binding to Sp1 (Chun *et al.*, 1998), stabilization of the TFIID/TFIIA complex on the HIV-1 TATA box (Kashanchi *et al.*, 1996), recruitment of a functional TBP or TFIID (Chiang and Roeder, 1995; Dal Monte *et al.*, 1997; Garcia-Martinez *et al.*, 1997; Kashanchi *et al.*, 1996; Roebuck *et al.*, 1997; Veschambre *et al.*, 1995), phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) by a number of kinases, including TFIIH (Blau *et al.*, 1996; Herrmann and Mancini, 2001; Parada and Roeder, 1996), and binding of Tat directly to RNA Pol II. In recent years, Tat has also been shown to bind a number of other factors regulating chromatin structure located at the HIV promoter and enzymes that phosphorylate the large subunit of RNA Pol II, resulting in efficient elongation of transcription. They include Tat/cyclin T/CDK9 and Tat/CBP/p300 (Bieniasz *et al.*, 1999; Deng *et al.*, 2000, 2001; Herrmann and Mancini, 2001).

Although much of the focus has been on Tat's ability to recruit the cyclin T/CDK9 complex [known as phosphorylated positive transcription

TABLE I Protein–Protein Interactions Between HIV-1 Viral Proteins and Their Cellular-Interacting Proteins[a]

Viral protein category	*HIV-1 virus protein*	*Cellular-interacting proteins*
Structural	Gag (p55)	
	p17—Matrix	BAF (Lin and Engelman, 2003; Mansharamani *et al.*, 2003), Calmodulin (Daube *et al.*, 1991; Radding *et al.*, 2000), HEED (Peytavi *et al.*, 1999), EF1α (Cimarelli and Luban, 1999), hIF2 (Wilson *et al.*, 1999), Actin (Bukrinskaya *et al.*, 1998)
	p24—Capsid	CypPA (Luban *et al.*, 1993)
	p7—Nucleocapsid	Actin (Cimarelli and Luban, 1999; Liu *et al.*, 1999)
	p6	Tsg101 (Garrus *et al.*, 2001; VerPlank *et al.*, 2001), AIP1 (Strack *et al.*, 2003)
	Pol	
	p51—Reverse transcriptase	β-Actin (Liu *et al.*, 1999)
	p31—Integrase	Importin/Karyopherin α/β (Gallay *et al.*, 1997; Hottiger and Nabel, 1998), INI1 (Gallay *et al.*, 1997), UDG (Yung *et al.*, 2001)
	Env (gp160)	ApoH (Willetts *et al.*, 1999)
	gp120	CD4 (McDougal *et al.*, 1986)
Accessory	Vpu (p16)	BTrCP (Margottin *et al.*, 1998), UBP (Callahan *et al.*, 1998), Fas (Casella *et al.*, 1999)
	Vpr (p12/p10)	TFIIB (Agostini *et al.*, 1996), Cyclin T1/CDK9 (Sawaya *et al.*, 2000), hVIP/mov34 (Mahalingam *et al.*, 1998), Karyopherin α (Gallay *et al.*, 1997), ANT (Jacotot *et al.*, 2001), HHR23A (Withers-Ward *et al.*, 1997), p300 (Felzien *et al.*, 1998), p53 (Sawaya *et al.*, 1998), UNG (Bouhamdan *et al.*, 1996), RIP/VprBP (Zhang *et al.*, 2001)
	Vif (p23)	Sp140 (Madani *et al.*, 2002), Vimentin (Karczewski and Strebel, 1996), Cul5 (Yu *et al.*, 2003), Elongin B/C (Yu *et al.*, 2003), Rbx1 (Yu *et al.*, 2003), CEM15/APOBEC3G (Sheehy *et al.*, 2003)
	Nef (p27/p25)	NBP1 (Lu *et al.*, 1998), Human thioesterase II (Watanabe *et al.*, 1997), CD4 (Grzesiek *et al.*, 1996), ASK1 (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003), AP-1, 2, 3 (Coleman *et al.*, 2006; Craig *et al.*, 2000; Greenberg *et al.*, 1997; Schwartz *et al.*, 1996), MHC-I (Schwartz *et al.*, 1996; Williams *et al.*, 2002), PI3K (Wolf *et al.*, 2001), β-COP (Piguet *et al.*, 1999), PACS1 (Piguet *et al.*, 2000), Src family tyrosine kinases (Lee *et al.*, 1996), p53 (Greenway *et al.*, 2002)

(*continued*)

TABLE I (*continued*)

Viral protein category	*HIV-1 virus protein*	*Cellular-interacting proteins*
Regulatory	Tat (p16/p14)	TBP (Chiang and Roeder, 1995; Dal Monte *et al.*, 1997; Garcia-Martinez *et al.*, 1997; Kashanchi *et al.*, 1994; Roebuck *et al.*, 1997; Veschambre *et al.*, 1995), p32 (Fridell *et al.*, 1995), CBP/p300 (Col *et al.*, 2001; Deng *et al.*, 2000, 2001; Hottiger and Nabel, 1998; Kiernan *et al.*, 1999; Ott *et al.*, 1999), CAK/TFIIH (Blau *et al.*, 1996; Cujec *et al.*, 1997; Herrmann and Mancini, 2001; Parada and Roeder, 1996), Tip60 (Kamine *et al.*, 1996), P-TEFb (Chen *et al.*, 1999; de Falco and Giordano, 1998; Fujinaga *et al.*, 1998; Herrmann and Mancini, 2001; Karn, 1999; Majello *et al.*, 1999; Wei *et al.*, 1998), Sp1 (Chun *et al.*, 1998; Kamine *et al.*, 1991), BRG1 (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006; Mohrmann *et al.*, 2004)
	Rev (p19)	p32 (Tange *et al.*, 1996), eIF-5A (Ruhl *et al.*, 1993), CRM1 (Neville *et al.*, 1997), hRIP/Rab (Farjot *et al.*, 1999), Importin-β (Truant and Cullen, 1999), B23 (Fankhauser *et al.*, 1991)

[a]Abbreviations: BAF, barrier-to-autointegration factor; HEED, human EED; EF1α, elongation factor-1α; hIF2, a human homologue of bacterial translation initiation factor 2; CyPA, cyclophilin A; Tsg101, tumor susceptibility gene 101; AIP1, actin-interacting protein 1; INI1, integrase interactor 1; RIP, receptor-interacting protein; NBP1, Nef-binding protein-1; ASK1, apoptosis signal-regulating kinase 1; AP, adaptor proteins; MHC, major histocompatibility complex; PI3K, phosphatidylinositol 3-kinase; PACS, phosphofurin acidic cluster sorting protein; P-EFBb, phosphorylated positive transcription elongation factor b.

elongation factor b (P-TEFb)], emerging work indicates the importance of Tat in recruiting other factors required for initiation and elongation. Several reports have indicated a role of Tat in associating with TBP and facilitating transcriptional initiation (Brady and Kashanchi, 2005; Chiang and Roeder, 1995; Kashanchi *et al.*, 1994; Raha *et al.*, 2005). Additionally, it has been shown that Tat associates with CDK2/cyclin E, promoting phosphorylation of Tat and the CTD of RNA Pol II (Ammosova *et al.*, 2006; Deng *et al.*, 2002).

1. Tat and Cyclin T/CDK9

Tat activates the HIV LTR by binding to TAR to recruit and activate cellular factors. TAR is a 59-residue RNA leader sequence that folds into a specific stem-loop structure (Bannwarth and Gatignol, 2005; Cullen, 1991; Garcia *et al.*, 1989). The sequence of the bulge (Cordingley *et al.*, 1990; Dingwall *et al.*, 1990; Roy *et al.*, 1990) and loop (Berkhout and Jeang, 1989; Feng and Holland, 1988; Selby *et al.*, 1989) of TAR is critical for Tat activation of the LTR. Tat binds to the bulge and recruits cellular factors that bind the loop, one of which, P-TEFb (Fig. 1C), a protein kinase composed of CDK9 and cyclin T1, is stimulated by Tat (Richter *et al.*, 2002; Wei *et al.*, 1998). Activation of P-TEFb results in hyperphosphorylation of the large subunit of the RNA Pol IICTD and activation of transcription elongation (Kim *et al.*, 2002).

CDK9 is analogous to a component of the P-TEFb, isolated from Drosophila, which stimulates promoter-paused RNA Pol II to enter into productive elongation (Chen *et al.*, 1999; de Falco and Giordano, 1998; Herrmann and Mancini, 2001; Karn, 1999; Majello *et al.*, 1999; Wei *et al.*, 1998). A histidine-rich stretch of cyclin T1, the other component of P-TEFb, binds to the CTD of RNA Pol II, which is required for the subsequent expression of full-length transcripts from target genes (Taube *et al.*, 2002). CDK9 phosphorylation is required for high-affinity binding of Tat/P-TEFb to TAR. Furthermore, P-TEFb phosphorylation regulates Tat transactivation *in vivo* (Fong and Zhou, 2000; Garber *et al.*, 2000; Zhou *et al.*, 2000). Other studies on P-TEFb-regulated transcription and the involvement of Tat reveal the involvement of multiple factors in transcriptional control. Other studies show the involvement, although not the requirement, of hSpt5 in Tat-mediated transcription (Winston, 2001; Wu-Baer *et al.*, 1998). Other studies have identified a cellular inhibitor of P-TEFb, called Hexim1 (Fraldi *et al.*, 2005; Schulte *et al.*, 2005), which inhibits P-TEFb in the presence of the 7SK small nuclear RNA (Chen *et al.*, 2004; Michels *et al.*, 2004). Interestingly, the ability of Hexim1 to inhibit Pol II transcription relies on the presence of a Tat-like arginine-rich motif (Yik *et al.*, 2004). These multiple levels of transcriptional control suggest that there are many nuances to Tat-induced transactivation that have not yet been discovered.

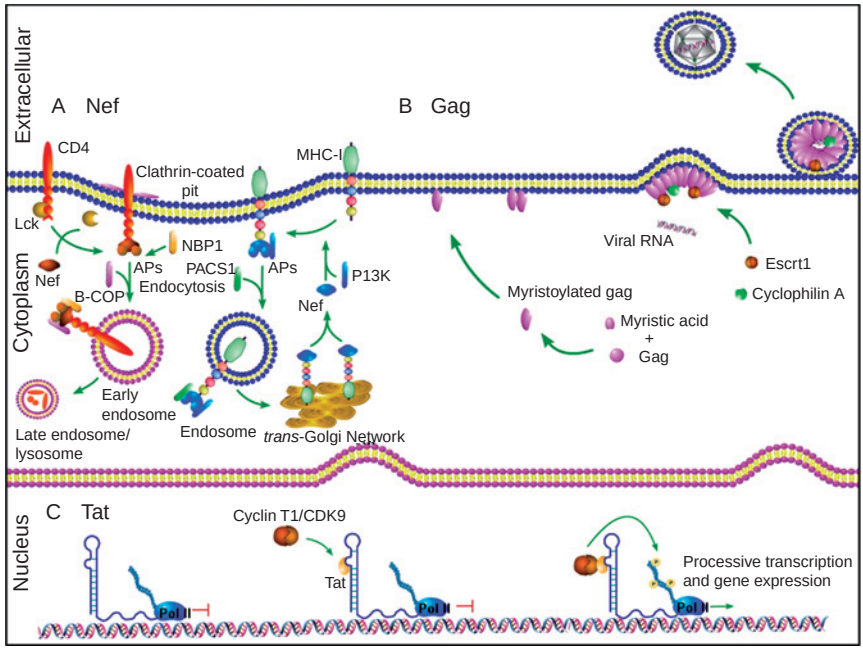


FIGURE 1 Alteration and subversion of cellular processes by *human immunodeficiency virus 1* (HIV-1). (A) Nef removes CD4 and major histocompatibility complex (MHC) from the cell surface by endocytosis and degradation. Nef competitively binds the C-terminal cytoplasmic tail of CD4, displacing bound Lck. AP allow binding of β-COP1, which binds to CD4-bound Nef, facilitated by binding of Nef-binding protein-1 (NBP1). β-COP1 then drives formation of the early endosome and finally the Nef complex dissociates from CD4 as it is further degraded in the lysosome. For MHC, binding of Nef to PI3K allows binding of Nef to MHC-I. The endocytosis of MHC-I is aided through the binding of phosphofurin acidic cluster sorting protein (PACS1). MHC-I is transported to the *trans*-Golgi Network where degradation of MHC and dissociation of Nef occur. (B) Coordinated modification of Gag followed by Gag–Gag and other cellular interactions leads to viral assembly and budding. Gag is myristoylated, which targets it to the cellular membrane. Once on the membrane, Gag interacts with other Gag molecules and begins to form the viral core. As this happens, Gag recruits viral RNA and the cellular genes cyclophilin A (CyPA) and Escrt1, which facilitate budding. (C) HIV-1 transcription is aided by Tat's ability to recruit cyclin T1/CDK9 [known as phosphorylated positive transcription elongation factor b (P-TEFb)], which in turn phosphorylates the C-terminal domain of RNA polymerase II (RNA Pol II). In the absence of Tat, transcription pauses between 50 and 100 nucleotides after transcription starts. In the presence of Tat, the TAR element recruits HIV-1 TAT, which recruits cyclin T1/CDK9, this in turn phosphorylates Pol II and leads to activated transcription.

2. *Tat and CBP/p300*

Examination of defective Tat transactivation in murine cells revealed a role for P-TEFb (Benkirane *et al.*, 1998; Bieniasz *et al.*, 1999; Fujinaga *et al.*, 1999; Ramanathan *et al.*, 1999). Additionally, studies of Tat transactivation

in murine cells revealed a role for p300 and PCAF. Tat has been shown to interact with the histone acetyltransferases (HAT) p300 and PCAF. Supporting evidence suggests that Tat-associated HAT activity is important for transactivation of integrated, but not unintegrated, HIV-1 (Deng *et al.*, 2000). Furthermore, it has been shown that the Tat–p300 interaction increases the HAT activity of p300 on histone H4, which is associated with nucleosomal DNA (Deng *et al.*, 2001). The association of Tat with p300 and PCAF may be critical for remodeling of the chromatin downstream of TAR to allow progressive transcription elongation. The acetyltransferase activity of p300 may also alter the activity of the viral Tat protein. p300 has been shown to acetylate lysine 50 in the TAR RNA-binding domain of Tat, while PCAF acetylates lysine 28 in the activation domain of Tat (Col *et al.*, 2001; Kiernan *et al.*, 1999; Ott *et al.*, 1999). Acetylation of lysine 28 by PCAF enhances Tat binding to the Tat-associated kinase, cdk9, while acetylation by p300 at lysine 50 of Tat promotes the dissociation of Tat from TAR RNA (Deng *et al.*, 2000; Kiernan *et al.*, 1999; Mujtaba *et al.*, 2002). Acetylation of lysines 28 and 50 of Tat has been demonstrated to be important for viral replication (Bres *et al.*, 2002; Deng *et al.*, 2000).

In support of the ability of acetylated Tat to increase transcription, several groups have demonstrated that SWI/SNF binds to acetylated Tat (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006; Treand *et al.*, 2006). SWI/SNF is a chromatin remodeling complex that may be involved in the removal of nucleosomes at the HIV-1 LTR and activates an increase in viral transcription (Agbottah *et al.*, 2006). This recruitment of SWI/SNF by Tat has been reported to be regulated by the binding of acetylated Tat to Brm (Treand *et al.*, 2006) or BRG1 (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006). This interaction was shown to be critical for achieving high levels of viral transcription.

B. Cellular Protein Interactions of Nef

The HIV-1 gene product Nef (negative factor) is a multifunctional protein that contributes to HIV pathogenesis through alteration of endocytosis, signal transduction via the downregulation of cell surface receptors, vesicular trafficking, and immune evasion; therefore, enhancing virion infectivity and viral production (Arold and Baur, 2001; Das and Jamel, 2005; Geyer *et al.*, 2001; O'Neill *et al.*, 2006; Roeth and Collins, 2006). Nef is a small protein of ~27 kDa that is expressed abundantly in the early stages of viral replication and regularly undergoes posttranslational modifications including phosphorylation and the irreversible myristoylation of its N-terminus; this localizes the protein to the cellular membrane (Arold and Baur, 2001; Bentham *et al.*, 2006; Geyer *et al.*, 2001; Harris, 1995). The structure of Nef includes a membrane anchoring region in addition to an unstructured

flexible loop providing an extensive surface area that is capable of undergoing important conformational changes (e.g., for transient binding) and is readily accessible for interactions (Arold and Baur, 2001; Geyer *et al.*, 2001). Nef has a positive effect on viral infection and replication by promoting the survival of infected cells through interaction with cellular proteins involved in both trafficking of cell surface receptors and signaling molecules (Fig. 2) (Arold and Baur, 2001; Das and Jamel, 2005).

1. Downregulation of CD4

CD4 is the primary host cell surface receptor required for T-lymphocyte ontogeny, activation of mature helper T lymphocytes, and serves as the primary receptor for HIV-1. Nef downregulates the transmembrane glycoprotein CD4 through the acceleration of endocytosis and lysosomal degradation in order to facilitate increased release and infectivity of virus particles as well as preventing superinfection (Arold and Baur, 2001; Das and Jamel, 2005; Geyer *et al.*, 2001; Watanabe *et al.*, 1997). Ironically, although CD4 is needed for viral infection, three of nine viral genes are involved in its removal and degradation shortly after infection (Bour *et al.*, 1999; Cortes *et al.*, 2002). Nef downregulation of CD4 prevents the formation of complexes between the HIV Env-encoded protein gp120 and CD4 (Arold and Baur, 2001; Benson *et al.*, 1993). It recruits additional adaptor proteins (APs) and cofactors in order to effectively internalize CD4. Nef directly binds the dileucine motif of the cytoplasmic tail of CD4 (Fig. 1A), therefore displacing the otherwise bound Src family tyrosine kinase Lck, and promotes the recruitment of AP-1, 2, 3 via the interaction of its $\mu 2$ subunit with Nef's own dileucine motif (Das and Jamel, 2005; Greenberg *et al.*, 1997; Mangasarian *et al.*, 1997; Piguet *et al.*, 1999). These AP complexes interact with cytosolic clathrin-coated pits that mediate transport between the *trans*-Golgi, endosomes, and lysosomes as well as vesicles that mediate endocytosis of CD4 (Craig *et al.*, 2000; Das and Jamel, 2005; Janvier *et al.*, 2001). The direct interaction of Nef and AP-2 is mediated and strengthened by the binding of Nef to Nef-binding protein-1 (NBP1) (Das and Jamel, 2005; Lu *et al.*, 1998). As a result, Nef targets these internalized CD4 molecules for degradation by both binding CD4 and recruiting β-COP, the β-subunit of COP1 coatomers in endosomes, for routing and shuttling of CD4 to lysosomes based on acidic residues (Piguet *et al.*, 1999). This degradation process is also facilitated by the binding of Nef to human thioesterase II protein that regulates the intracellular level of acyl-CoA therefore increasing the efficiency of protein myristoylation, which is critical for the membrane localization of Nef (Liu *et al.*, 1997; Watanabe *et al.*, 1997). These interactions occur quickly, as Nef, once localized to the cell membrane, is endocytosed with CD4 within minutes (Arold and Baur, 2001).

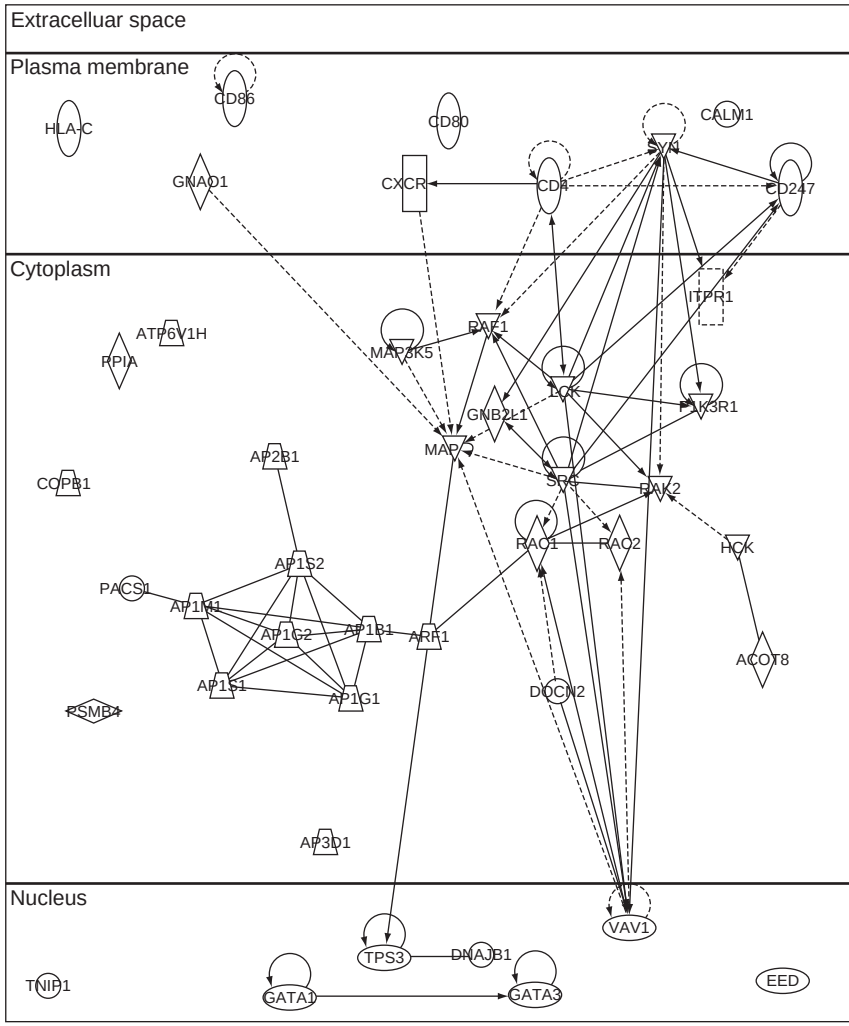


FIGURE 2 System diagram of Nef-interacting proteins. Ingenuity generated diagram showing the relationship between various Nef-binding proteins. The close interaction of these proteins suggests a coordinated effect by Nef upon the host cell. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

2. Downregulation of MHC-I

Mobilization of the host's adaptive immune response to a viral infection involves the presentation of viral peptides on the surface of infected cells by the major histocompatibility complex (MHC). The downregulation of

MHC-I by Nef assists HIV-1-infected cells in escaping the cytotoxic T-lymphocyte-mediated elimination of virus-infected cells (Das and Jamel, 2005; Geyer *et al.*, 2001). Nef increases the rate of internalization of cell surface MHC-I molecules through the endosomes (via AP-1 binding) followed by transportation to the *trans*-Golgi network, then finally to clathrin-containing vesicles (Fig. 1A) (Das and Jamel, 2005; Roeth *et al.*, 2004). Nef selectively downregulates the HLA-A and HLA-B MHC-I molecules through binding a unique cytoplasmic tyrosine kinase residue, as opposed to the HLA-C and HLA-E molecules, which are required for cellular protection from lysis by natural killer cells (Das and Jamel, 2005). The Nef shuttling effect on MHC-I to the *trans*-Golgi network is blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K) through the additional binding of phosphofurin acidic cluster sorting protein (PACS1) (Das and Jamel, 2005; Peterlin and Trono, 2003; Piguet *et al.*, 2000; Swann, 2001; Wolf *et al.*, 2001). Nef, therefore, decreases the expression of MHC-I molecules by manipulating PACS1 that controls the endosomes-to-Golgi trafficking of furin and M6PR by bridging those molecules with the AP complex of endosomal clathrin-coated pits (Peterlin and Trono, 2003; Piguet *et al.*, 2000).

3. Nef and Apoptosis

Nef interferes with the Fas-FasL apoptotic pathway by inhibiting apoptosis signal-regulating kinase 1 (ASK1), a serine/threonine kinase significant in both the Fas and the tumor necrosis factor (TNF) signaling pathways (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003). The binding of Nef to ASK1 inhibits both Fas- and TNF-α-mediated apoptosis (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003). Additional Nef interactions affecting apoptosis include the binding and activation of PI3K, PI3K phosphorylates PAK, PAK phosphorylates Bad, resulting in the release of the antiapoptotic Bcl-2 or Bcl-X_L complex (Das and Jamel, 2005; Peterlin and Trono, 2003). Nef also interacts directly with tumor suppressor protein p53, decreasing its proapoptotic, transcriptional, and DNA-binding activities, and protecting HIV-1-infected cells from p53-mediated apoptosis (Das and Jamel, 2005; Greenway *et al.*, 2002). Blocking and interfering with these apoptotic pathways can prevent the premature death of an HIV-1-infected cell by virus-induced cytopathicity that leads to the completion of the viral replication cycle (Peterlin and Trono, 2003).

C. Cellular Protein Interactions of Gag

HIV-1 Gag drives the assembly and release of infectious viral particles during the final stage of viral replication (Freed, 1998; Gottlinger, 2001; Li and Wild, 2005). HIV-1 Gag is synthesized initially into a precursor polyprotein, $Pr55^{Gag}$, and is subsequently cleaved shortly after budding by

the HIV-1 protease into the mature Gag proteins: p17 matrix, p24 capsid, p7 nucleocapsid, and p6 (Freed, 1998). Maturation of the Gag proteins results in specific localization throughout the virion as well as major morphological transformation of the virion structure (Freed, 1998). The ability of Gag to interact with various cellular proteins is important to its function in both infection of a target cell and maturation of the virus (Fig. 3). HIV-1 Gag

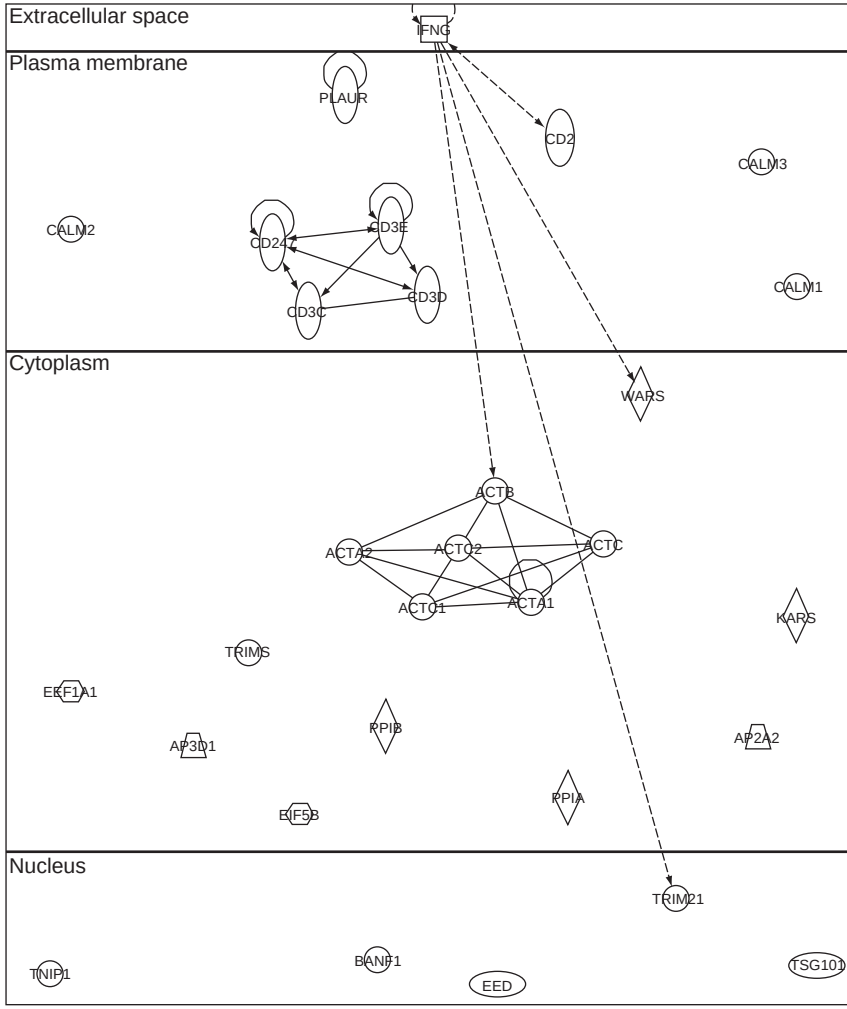


FIGURE 3 System diagram of Gag-interacting proteins. Ingenuity generated diagram showing the relationship between various Gag-binding proteins. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

proteins, in addition to integrase and viral nucleic acids, are incorporated into PICs along with cellular proteins in order to actively penetrate the nuclear membrane initiating infection (Sorin and Kalpana, 2006). Upon infection, the HIV-1 PICs rapidly associate with the cytoskeletal compartment through specific protein–protein interactions between viral factors and host cellular proteins (Tasara *et al.*, 2001).

1. p17 Matrix: Directing Assembly and Binding at Plasma Membrane

In mature virions, the matrix protein p17 is a 132-amino acid polypeptide derived from the N-terminus of the $Pr55^{Gag}$ precursor that forms a protective shell attached to the inner surface of the plasma membrane of the virus (Fiorentini *et al.*, 2006). The major function of p17 is to direct binding and assembly at the plasma membrane of the virion (Freed, 1998). Localization of p17 is determined via posttranslational modifications where myristoylation targets the plasma membrane and additional phosphorylation facilitates release from the membrane to associate with reverse transcription complexes (Bukrinskaya *et al.*, 1998). Calmodulin binding to the membrane-binding amphipathic region of p17 also regulates matrix localization mimicking the effect of proteolysis (Radding *et al.*, 2000). The matrix protein binds actin microfilaments of the host cell cytoskeleton after infection to activate reverse transcription (Bukrinskaya *et al.*, 1998; Tasara *et al.*, 2001). Barrier-to-autointegration factor proteins are also incorporated into HIV-1 virions and interact with p17 to aid in the transition from reverse transcription complex to PIC as well as to interact with viral DNA (Mansharamani *et al.*, 2003). Additional transcriptional regulation is seen through the binding of p17 to the human EED (HEED) protein, a homologue of the mouse gene *eed* family that have been reported to function as transcriptional repressors and gene silencers (Peytavi *et al.*, 1999). It is hypothesized that HIV-1 infection might deregulate silent cellular genes or that HEED might be involved in the docking of the PIC to specific host DNA insertion sites via p17 binding (Peytavi *et al.*, 1999). p17 alters translational efficiency as well as transcriptional via binding of elongation factor-1α, essential for the delivery of aminoacyl-tRNAs to ribosomes (Cimarelli and Luban, 1999). The accumulation of Gag p17 impairs translation and therefore serves to release viral RNA from polysomes (Cimarelli Luban, 1999). The direct binding of the human homologue (hIF2) of bacterial translation initiation factor 2 also has the potential to regulate viral translation (Wilson *et al.*, 1999).

2. p24 Capsid and p7 Nucleocapsid

The capsid protein p24, consisting of an N- and a C-terminal domain, condenses to form the conical core structure surrounding the viral genome (Endrich *et al.*, 1999). The predominant interaction of p24 is with cellular

protein cyclophilin A (CyPA), which characteristically has a peptidyl-prolyl *cis-trans* isomerase activity and interacts with cyclosporin A (Endrich *et al.*, 1999). The precise role of CyPA in the viral life cycle remains elusive; however, the knockdown of CyPA expression or the disruption of the p24–CyPA interaction results in reduced infectivity and prevents encapsidation into the virion (Sorin and Kalpana, 2006). Virions depleted of CyPA are blocked at early stages of reverse transcription, suggesting involvement in the early stages of infection (Sorin and Kalpana, 2006). The p7 nucleocapsid protein plays several important roles in the viral life cycle including virus assembly, viral genomic RNA encapsidation, primer tRNA placement, and enhancement of viral reverse transcription (Liu *et al.*, 1999). p7 can bind actin directly therefore associating the HIV-1 Gag protein with the cytoskeleton (Liu *et al.*, 1999).

3. p6: Accessory Protein

Efficient HIV release requires the *cis*-acting, tetrapeptide P(S/T)AP "late domain" (L domain) found in the p6 domain of Gag (von Schwedler *et al.*, 2003). The L domain mediates the detachment of the virion by recruiting host tumor susceptibility gene 101 (Tsg101), a component of the class E vacuolar protein sorting (Vps) machinery (Strack *et al.*, 2003), which is involved in regulation of intracellular trafficking, transcriptional regulation, and cell cycle control (VerPlank *et al.*, 2001). Binding of p6 to Tsg101 is required for the release of infectious HIV-1 (Fig. 1B) and facilitates budding by linking the p6 L domain to vacuolar sorting machinery (Garrus *et al.*, 2001). A second region within p6 contributes to the virus release function through the binding of host protein, ALG-2-interacting protein 1, serving as a component of the viral budding machinery (Strack *et al.*, 2003).

V. Viral-Induced Cellular Alterations

A. Tat: Effects on Cellular Transcription

The primary function of Tat is the transactivation of the viral LTR through recruitment of cellular factors to the viral promoter. By interacting with TAR, Tat recruits cyclin T1/CDK9 and activates Pol II-driven transcription (Fig. 1C). In addition, Tat interacts with CBP/p300 that remodels chromatin for more efficient transcription, the recruitment of which is regulated by the ability to bind to nascent RNA transcripts. Tat binding to cellular targets provides a mechanism with which the virus can alter the environment favorably. Given Tat's specific interactions with transcription factors such as Sp1, TBP, and P-TEFb, among others, it has the ability to alter gene expression at the transcriptional level; indeed, many studies have shown that Tat expression influences native cellular processes

(Chauhan *et al.*, 2003; Coiras *et al.*, 2006; de la Fuente *et al.*, 2002; Ensoli *et al.*, 1993; Izmailova *et al.*, 2003; Liang *et al.*, 2005; Nelson *et al.*, 2003; New *et al.*, 1997; Pocernich *et al.*, 2005; Viscidi *et al.*, 1989; Westendorp *et al.*, 1994). In order to further examine the role of Tat in regulation of cellular processes, several groups have chosen to use a proteomic or genomic approach.

Dendritic cells (DCs) are likely the first cells infected following mucosal exposure to HIV-1. Izmailova *et al.* (2003) examined the changes in Tat-expressing DCs using Affymetrix gene arrays. They found that Tat upregulated many interferon-inducible genes. Specifically, Tat upregulated the expression of interferon regulatory factor-7 and signal transducer and activator of transcription 1 (STAT1), both of which are transcriptional regulators of interferon response and may be responsible for upregulation of other IFN-inducible genes (Izmailova *et al.*, 2003). Additionally, four chemokines were shown to be upregulated in Tat-expressing DCs: human monokine induced by interferon-γ, monocyte chemoattractant protein-3, monocyte chemoattractant protein-2, and interferon-inducible protein-10. In support of these findings, the authors show that culture supernatant from Tat-expressing DCs induced monocyte and T-cell chemotaxis. Interestingly, despite these changes in function and chemokine production, DCs expressing Tat did not become activated or differentiate into mature DCs, as determined by detection of the cell surface markers CD40, CD80, CD83, CD86, and CD25 (Izmailova *et al.*, 2003).

In a similar study, the effects of Tat on $CD4^+$ T cells, the primary target of HIV-1, was examined using a proteomic approach (Coiras *et al.*, 2006). Opposing studies have shown that Tat can induce apoptosis in uninfected bystander T cells; however, Coiras *et al.* (2006) proved the opposite effect for Tat-expressing cells. In fact, Tat induced resistance to apoptosis mediated by tunicamycin in Jurkat T cells. The authors associate this effect with the observed downregulation of cytoskeletal proteins such as β-actin and β-tubulin. In the absence of β-actin and β-tubulin, there is a disruption in cytoskeletal arrangement, leading to the loss of acting depolymerization as mediated by apoptosis-inducing signals, thus altering a critical event in the apoptotic cascade. The authors also observed the downregulation of Annexin II and the Rac/Rho-GDI complex, which influences the fusion and internalization of an infecting virion, thereby reducing the rate of super infection.

Another study examined the effects of Tat expression in human astrocytes, as a means to examine processes important in HIV-1-associated dementia (Pocernich *et al.*, 2005). This study also observed downregulation of β-tubulin and components of the Rac/RHO-GDI complex. Other proteins downregulated in Tat-expressing astrocytes included protein phosphatase 2A (PP2A) inhibitor, heterogeneous nuclear ribonucleoprotein A1, and heat-shock protein 70 (HSP70), which all likely cause an increase in viral replication. PP2A augments Tat-regulated transcription (Ammosova *et al.*, 2005; Faulkner *et al.*, 2003), therefore a downregulation of PP2A inhibitor will

increase transcription. Heterogeneous nuclear ribonucleoprotein A1 may also downregulate Tat-regulated transcription or block splicing and production of the Tat protein. HSP70 downregulation, along with HSP32, protects the cell from stress-induced cell death. Finally, HSP70 has been shown to be involved in chaperoning and allow the correct folding of the cyclin T/CDK9 complex; suggesting that a downregulation of HSP70 may affect P-TEFb activity (O'Keeffe *et al.*, 2000).

Two papers published by de la Fuente *et al.* (2002) and Liang *et al.* (2005) examine the effect of Tat on cellular gene expression in T-cell lines, focusing specifically on latently infected cells as well as the differential effects of Tat at various stages of the cell cycle. Interestingly, the microarray experiments performed in these studies showed that ~66% of the differentially expressed genes were downregulated. de la Fuente *et al.* (2002) identified many genes differentially regulated in latently infected T cells as compared to uninfected cells. Clustering of the differentially expressed genes identified four distinct cellular networks: signal transduction, translation, cell cycle regulation, and chromatin remodeling. Overall, the observed changes suggest that the Tat-expressing cells are altered to express larger amounts of virus through chromatin remodeling and an increase in translational machinery. In addition, signal transduction and cell cycle machineries are altered in a way that favors proliferation and survival of the cell (Fig. 4).

A subsequent study by Liang *et al.* (2005) examined the Tat-dependent effects in different stages of the cell cycle in an attempt to identify therapeutic targets in the host cell genome. The authors observed many of the same differentially expressed genes as previous studies, however, choose to focus on a subset of genes whose upregulation due to Tat is independent of the cell cycle. The role of Rev-binding protein 2, Pou2Af1, cyclin A1, PPGB, EXT2, and HEXA was examined using small interfering RNAs (siRNA)-mediated knockdown. siRNA-mediated repression of these genes did not result in changes in the cell cycle or induction of apoptosis; however, knockdown of these genes resulted in a lower level of p24 Gag detectable in the cell culture media, suggesting a drop in viral replication (Liang *et al.*, 2005). This work showed evidence that viral replication can be blocked by targeting cellular proteins critical to viral replication.

Each of these studies outlines a cellular phenotype associated with Tat expression. An emerging pattern of Tat-influenced differentially expressed cellular genes provides insight into the effect of Tat expression. Tat functions through an upregulation of gene expression; however, 66% of cellular genes are downregulated in a Tat-expressing cell, likely through a secondary effect. For instance, Tat activates specific interferon response genes, prevents apoptotic signaling, triggers proliferation, promotes cellular chemotaxis, triggers release of chemoattractants, and prevents differentiation. When taken together, these changes indicate that Tat is providing a favorable environment for virus production. Resistance to apoptosis and the proliferative

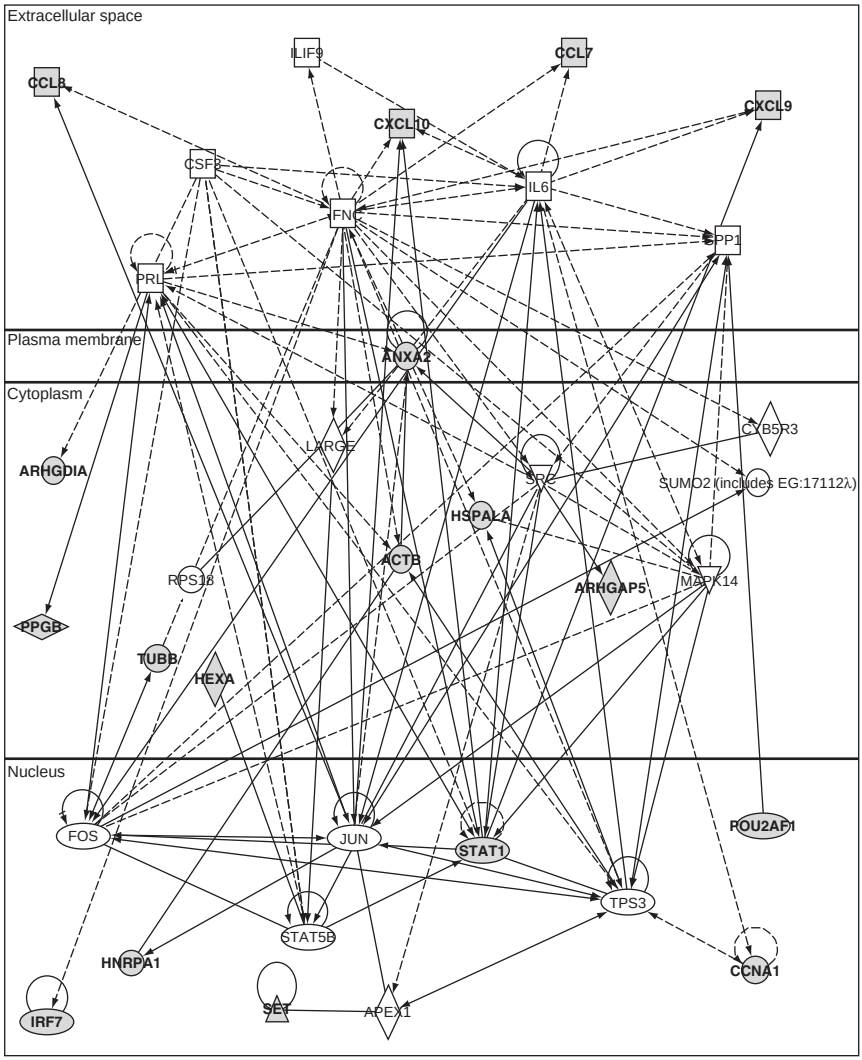


FIGURE 4 System diagram outlining Tat's effect on cellular proliferation. Ingenuity generated diagram showing the relationship between 18 genes differentially regulated by Tat (gray shaded nodes) and other genes relating to cellular proliferation. This diagram shows how alteration of expression of a few key genes can have a large effect on the cellular system. Of particular interest are the more important nodes such as STAT1, Annexin II, and Fos. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

signals produced by Tat ensure that viral replication and transcription continue. Meanwhile, the changes in chemokine expression and cellular migration ensure that target cells are available to be infected by the newly

produced virions. The function of Tat in altering the cellular environment provides a target for therapy.

Flavopiridol, originally developed as an anticancer agent, functions by blocking cyclin T1/CDK9 function. It is highly specific for cdk9 inhibition and can be used to block Tat transactivation of viral LTR at a very high therapeutic index (Chao *et al.*, 2000; de la Fuente *et al.*, 2003; Sedlacek, 2001). This ability to block Tat transactivation through P-TEFb may also allow the drug to prevent the alteration of cellular gene expression by Tat.

A study by Nelson *et al.* (2003) examines the ability of flavopiridol not only to block Tat transactivation of the viral LTR but also to diminish some of the pathogenic effects induced by HIV-1 gene expression. Using a murine model of HIV-1-associated nephropathy (HIVAN) that focuses on renal expression of full-length and spliced HIV-1 mRNAs from a Gag and Pol-deficient provirus, the authors examine the effects of flavopiridol treatment on viral gene expression, renal pathogenesis, cellular gene expression, and toxicity. Proviral gene expression was reduced in all flavopiridol-treated animals, but not in control animals. Several clinical parameters of HIVAN were also examined in flavopiridol-treated animals (Nelson *et al.*, 2003). The ratio of urine protein to creatinine levels normalized in flavopiridol-treated animals. Additionally, four serological abnormalities associated with HIVAN (albumin levels, blood urea nitrogen, cholesterol, and triglycerides) returned to normal after flavopiridol treatment. Liver pathology, as determined by histochemical analysis, also returned to normal in treated animals. These results were traceable to the gene expression level through microarray analysis. Microarrays indicated that genes differentially expressed in HIV-expressing liver were returned to levels comparable to healthy liver (Nelson *et al.*, 2003).

This study shows the feasibility of targeting cellular genes involved in viral replication (Nelson *et al.*, 2003). By approaching possible treatment options through inhibiting Tat transactivation, the authors have identified a way to both block viral replication and treat the pathogenic effects associated with infection. This approach may provide a perfect complement to the current therapies that prevent the infection of new cells. The use of flavopiridol will reduce the production of infectious virus, as well as deplete the effects of viral infection. When applied to the primary target of infection, the immune system, this approach may possibly prevent much of the immune dysregulation associated with HIV-1 infection.

The association of Tat with cyclin E/CDK2 and subsequent phosphorylation of RNA Pol II CTD and Tat suggest an important role cdk2 in HIV-1 transcription (Ammosova *et al.*, 2006; Deng *et al.*, 2002). As a direct extension of this work, two papers have been published that examine the use of Cyc202, a cyclin E/CDK2 inhibitor, in blocking HIV-1 replication (Agbottah *et al.*, 2005; Wang *et al.*, 2001). Previous work by these authors showed that p21/Waf1, a cellular inhibitor of cdk2, 3, 4, and 6, was downregulated in

HIV-1-infected cells (Clark *et al.*, 2000). They reasoned that HIV-1-infected cells might be more sensitive to a cdk inhibitor, such as Cyc202, than uninfected cells. The authors showed that Cyc202 both decreases the rate of HIV-1 transcription and causes selective apoptosis of infected cells. In this way, the authors have identified a viral-induced change (loss of p21/Waf1), observed a crucial interaction between virus and host (Tat/cyclin E/CDK2), and targeted this interaction with a specific drug.

B. Nef: Altering the Cell Surface

A microarray study examining the effects of Nef on T-cell activation has identified a number of cellular factors upregulated in Nef-expressing cells. Simmons *et al.* (2001) created a Jurkat T-cell line that expresses Nef under the control of the doxycycline transactivator. The authors identified a series of upregulated genes associated with T-cell activation; NFκB, Jun-D, cFos, TFIID, cdk9, and various Pol II subunits. Additionally, the study identified several genes upregulated by Nef that have been shown to be upregulated in infected primary cells such as HSP70. Overall, these changes are indicative of an activated T cell in which viral replication will be increased. Interestingly, these Nef-mediated changes required the presence of several T-cell receptor subunits, suggesting that this transcriptional profile was the result of Nef altering cell membrane associated factors and tuning the cell for maximum activation. Nef-expressing cells were also treated with the immunosuppressive drug cyclosporin A, which inhibited expression of genes upregulated by Nef, suggesting a role of NFAT and NFκB in the Nef-mediated alteration of the cellular activation (Simmons *et al.*, 2001).

A different study identified a role for Nef in altering cholesterol synthesis in the cell. Indeed, HIV-1 infection is inherently linked to cholesterol metabolism such that the virus buds from cholesterol-rich lipid rafts on the cell surface, as well as evidence that prolonged HIV disease is associated with a systemic lypodystrophy (van't Wout *et al.*, 2005). van't Wout *et al.* (2005) showed that HIV-1 infection mediates the upregulation of a panopoly of enzymes associated with cholesterol biosynthesis, including the enzyme involved in the rate-limiting step of cholesterol biosynthesis, HMGCR. This increase in factors necessary for cholesterol biosynthesis was not present in cells infected with a virus lacking Nef. Additionally, increased cholesterol production was detected in cells infected with wild-type, but not δ-Nef, viruses. This lack of cholesterol production was associated with a decrease in the infectivity of progeny virus, likely due to the absence of sufficient cholesterol in the lipid rafts.

A study by Janardhan *et al.* (2004) elucidates a possible mechanism for Nef's ability to alter cell signaling and trigger T-cell activation. This group used a Jurkat cell line expressing an HA and Flag-tagged Nef to identify Nef-interacting proteins. Nef associated proteins were then identified by MS

analysis. The most interesting finding was the association of Nef with the DOCK2/ELMO1 complex, which is necessary to Rac-induced signaling. Supporting data showed that Nef expression in resting T cells activates Rac signaling, which appears to reduce the T-cell activation threshold. In agreement with previous Nef studies, this chapter identifies a role for Nef in altering the membrane compartment of the cell and encouraging T-cell activation.

Although no drug currently exists to block Nef-mediated effects, the above studies suggest that Nef may provide a valuable therapeutic target. Indeed, Nef is dispensable for replication in tissue culture but is also needed for pathogenesis in native infection. This suggests a role for Nef in mediating the disease phenotype beyond its direct interaction with the virus. In support of Nef as a drug target, several groups have targeted siRNA against Nef and show reduction in viral replication—a result that agrees with studies that have used Nef-deficient viruses in their proteomic and genomic screening. The use of cyclosporin A reveals that it may be possible to counteract Nef-mediated changes in the cell by targeting naturally occurring signal cascades.

Nef directly manipulates the extracellular surface of the host plasma membrane in order to mediate optimal viral conditions for infection, budding, and release. The protein composition of the surface and cytoplasmic faces of the membrane directly influence the efficiency and success of any potential HIV-1 infection. The identification and characterization of membrane-associated proteins, therefore, would provide an accurate library of cell signaling receptors and would accentuate the infection and budding processes of HIV-1. Unfortunately, the development of such a membrane proteome encounters numerous difficulties in isolating membrane proteins away from other cellular organelles as well as from the membrane itself while still remaining intact. A study by Berro *et al.* (2007) has successfully identified and characterized the membrane proteome of HIV-1-infected cells using a biotin-directed affinity purification method. This study additionally utilized the identification of surface markers through comparative proteomics (2DGE and MALDI-TOF) between ACH2 HIV-1 latently infected T cells and its parental uninfected cell line CEM, which allowed for the characterization of the presence of differentially expressed proteins between these two states. Seventeen proteins were found to be differentially expressed on the membrane of ACH2 cells regulating cellular and viral pathways including cell survival, differentiation, apoptosis, adhesion, and migration. For example, the receptor-associated proteins that were identified as differentially expressed in ACH2 cells include Bruton's tyrosine kinase and the X-linked inhibitor of apoptosis. Both of these proteins are involved in antiapoptotic pathways and were found exclusively in infected cells, therefore it was hypothesized that infected cells upregulate antiapoptotic mechanisms to counterbalance virus-induced apoptosis and

maintain cell survival. Bruton's tyrosine kinase is a protein tyrosine kinase which, on activation, is translocated to the membrane where it mediates apoptosis through the activation of members of the antiapoptotic machinery, through the PI3K pathway, or through the NFκB pathway (Bajpai *et al.*, 2000; Berro *et al.*, 2007; Islam and Smith, 2000; Petro *et al.*, 2000, 2002). X-linked inhibitor of apoptosis inhibits caspases 3, 7, and 9 suppressing apoptosis indicating that during infection cells are dependent on antiapoptotic pathways to counter the apoptotic function of HIV-1 accessory protein (Berro *et al.*, 2007; Liston *et al.*, 1996; Schimmer *et al.*, 2004). Therefore, unique cell surface proteins present on HIV-1-infected cells could be utilized in antiviral therapy against an infection that would preferentially target a pathway vital for viral replication, but be dispensable for normal cellular function.

C. Gag: Using Cellular Factors to Facilitate Budding

Discrete functional domains within retroviral Gag proteins typically direct the association with plasma membranes, Gag–Gag dimerization, and the budding of virus from membranes driving the assembly and release of retroviral particles (Goila-Gaur *et al.*, 2003). L domains of Gag appear to promote viral budding by interacting with cellular host factors through binding of specific sequence motifs (Goila-Gaur *et al.*, 2003). These L domains have also been shown to associate with the cellular ubiquitination and endosomal sorting machinery of the host cell (Sorin and Kalpana, 2006). The identification of cellular host proteins critically involved in the cellular endosomal pathway that function late in the assembly/release pathway during the budding of virions from the plasma membrane through a combination of biochemical, virological, molecular, and structural approaches has provided insight into the manner in which viruses have evolved to exploit this cellular machinery (Freed, 2003).

The p6 domain of HIV-1 Gag contains a highly conserved PTAP motif that confers HIV-1 L domain activity and is critical for virus release (Goila-Gaur *et al.*, 2003; Reeves and Piefer, 2005). Lysine residues on HIV-1 p6 are monoubiquitinated serving to facilitate budding by targeting defective Gag molecules for proteolytic degradation, by preventing interference with viral budding, or to create docking sites for cellular proteins participating in viral budding (Garrus *et al.*, 2001). Monoubiquitination of proteins is also often a signal for sorting proteins from either the biosynthetic or the endocytic pathways to the multivesicular body and the lysosomes (Freed, 2003). As such, the human Tsg101, a ubiquitin conjugating E2 enzyme variant protein, binds to HIV-1 p6, shown through Y2H studies, indicating that a specific component of the cellular trafficking machinery is involved in retrovirus release (VerPlank *et al.*, 2001). Tsg101 is involved in regulation of intracellular trafficking, transcriptional regulation, and cell

cycle control and plays an essential role in forming and sorting protein cargo into the multivesicular body /late endosome (Freed, 2003; VerPlank *et al.*, 2001). Identification of a cleaved fragment of Tsg101 from a μRPLC-MS/MS analysis was published by Chertova *et al.* (2006). A proteomic analysis was performed to identify any and all cellular host proteins, which become incorporated into a budding endosomal virion (Chertova *et al.*, 2006). Serving as a method of both Tsg101 verification and proteomic analysis validation, the presence of Tsg101 in an endosomal virion confers confidence. Depleting Tsg101 or inhibiting endosomal trafficking also prevents HIV-1 release at a late stage, providing a direct correlation between lack of binding and function (Garrus *et al.*, 2001). Complementary siRNA studies have been performed to demonstrate the critical role of Tsg101 in the process of HIV-1 budding (Elbashir *et al.*, 2001; Garrus *et al.*, 2001; Tang, 2002). Garrus *et al.* (2001) designed a siRNA duplex homologous to a region of the Tsg101 coding sequence causing depletion of endogenous Tsg101 to undetectable levels. Depletion of Tsg101 using this method significantly reduced the release of virion-associated matrix and capsid proteins and reduced viral infectivity (Garrus *et al.*, 2001). An exogenous siRNA-resistant protein expression construct was introduced in Tsg101-depleted cells order and was shown to rescue viral release and infectivity (Garrus *et al.*, 2001; Tang, 2002). This assay validated the importance of the p6–Tsg101 interaction in HIV replication and due to no significant alteration of growth characteristics in Tsg101-depleted cells, it also demonstrated the importance of a potential target for novel antiviral agents (Tang, 2002). Unfortunately, any existing anti-budding treatments involving Tsg101 reduce viral infectivity significantly *in vitro*; however, encounter problems with cellular toxicity *in vivo* (Reeves and Piefer, 2005).

VI. Other Approaches

In addition to the typical proteomic or genomic approaches outlined above, which provide methods for broadly screening interactions, changes in transcription, and alterations in cellular proteins, the tools of the "omics" age may be utilized in other ways. These include examinations of the proteome of the virion, the identification of biomarkers, and the use of siRNA in treatment and screening.

A. Virion Proteome

Although standard proteomic approaches have been applied to monitor changes in the protein expression and localization in infected cells, these techniques may also be used to examine the content of the virion. As the virion buds from the cell, it incorporates cellular proteins from the plasma membrane and cytoplasm of the cell. These proteins may be incorporated at

random, based on the composition of the membrane and cytoplasm or they may be preferentially incorporated into the virion through associations with viral proteins. These proteins may be important to the viral life cycle and provide a target for future therapies.

Two notable studies examined the content of the virion using proteomic techniques (Chertova *et al.*, 2006; Saphire *et al.*, 2006). Both studies purified virion from infected cells and then identified virion-associated proteins by MS analysis. Chertova *et al.* (2006) evaluated the content of virions that bud internally in macrophages. In support of the validity of this method, they identified Tsg101 and actin-interacting protein 1, two cellular proteins whose interaction with Gag is necessary for virion budding. In this way, proteomic analysis of the virion may reveal the presence of proteins preferentially packaged during budding, which may be critical to viral budding, fusion, or integration.

The study by Saphire *et al.* (2006) examines the use of LC/tandem MS in identifying proteins associated with the virion. In validation of their technique, they successfully identified CyPA as a virion-associated protein. In addition, they identified CD48, a cellular complement determining factor, in the virion. This suggests a possible role for CD48 in avoiding complement mediated lysis. Interestingly, both of these groups identified histones within the virion. Although this might be due to cosedimentation of RNA-associated histones with the virion, this finding leads to the intriguing possibility that histones may be packaged with the virus. These studies outline the validity of using proteomic approaches in identifying virion components, but do not pursue the importance of their findings. However, future work in identifying proteins specifically incorporated into the virion may elucidate a new sphere of possible drug targets.

B. Biomarkers

A biomarker is a substance used to identify a particular biological state, especially a disease state such as cancer or infection. Although plasma viral load and antibody titer serve as efficient diagnostic markers in screening for infected individuals, there may be some value to identifying biomarkers associated with HIV-1 disease.

Small subsets of infected individuals, called long-term nonprogressors (LTNPs), naturally repress the virus (Guadalupe *et al.*, 2003; Paroli *et al.*, 2001; Valdez *et al.*, 2002). In these patients viral load becomes undetectable and CD4 T-cell counts are stable for 10 years or more. It is not currently well understood what mechanisms control viral replication in these individuals. Comparison of samples from LTNPs versus viremic patients may yield the discovery of biomarkers associated with nonprogression. Indeed, Sankaran *et al.* (2005) examined the gene expression profile of gut mucosal T cells in LTNPs as compared to patients with high viral load. They determined that

nonprogression was associated with a state of immune inactivation, which could potentially prevent the spread of virus. LTNPs represent an interesting subset of infected individuals. These patients are capable of living with the disease, in the absence of therapy, for long periods of time. Understanding the mechanisms and markers associated with LTNPs may yield clues as to how HIV-1 virulence is mediated. Studies such as these may help direct the focus of future studies concerned with controlling HIV-1 disease after infection.

A case study by Andersson *et al.* (2006) examines the value of using biomarkers to monitor the disease state in HIV-1-infected patients. They show that protein levels in the cerebrospinal fluid of infected patients can be used as a surrogate marker for HIV-1 associated dementia. Specifically, they show that treatment with highly active antiretroviral therapy reduces the levels of cerebrospinal fluid-associated NFL, Tau, and GFAP proteins, which have been previously associated with ongoing neural damage. Although studies such as these cannot be easily linked to therapy, biomarkers can be used to monitor the effectiveness of treatment and possibly provide clues to the mechanisms of disease pathogenesis.

C. RNA Interference

RNA interference (RNAi) is a recently discovered cellular pathway wherein short sequences of RNA can target genes for silencing through transcriptional repression, RNA cleavage, or translation inhibition (Agrawal *et al.*, 2003; Bartel, 2004; Hannon, 2002; Zamore and Haley, 2005). Study of the mechanisms of RNAi has made it possible to design siRNAs that will specifically silence a targeted gene. This discovery makes it possible to quickly develop siRNAs to alter the expression of a target gene. With this in mind, RNAi can be used to target the virus, cellular genes involved in viral replication, or target cellular genes to screen for involvement in RNAi.

A broad selection of studies have examined the efficacy of siRNA in treating HIV infection (Chang *et al.*, 2005; Colbere-Garapin *et al.*, 2005; Jacque *et al.*, 2002; Morris and Rossi, 2006b; Provost *et al.*, 2006; Suzuki *et al.*, 2005; Westerhout *et al.*, 2005). siRNA targeted against various sequences within the HIV genome have shown to be effective in suppressing viral replication (Jacque *et al.*, 2002; Morris and Rossi, 2006a,b). However, the high mutation rate of the virus allows the production of variants that are resistant to the administered siRNA. One strategy to overcome this is delivering siRNAs targeted to the wild-type sequence as well as the likely escape mutations (ter Brake and Berkhout, 2005). Unlike standard pharmaceuticals, the sequence specificity makes it possible to design siRNA to target any desired sequence. ter Brake *et al.* demonstrate that siRNA targeted against likely escape mutants can be effective. Various methods are being developed to allow delivery of siRNA to the appropriate target cell ranging

from lentiviral delivery to antibody-mediated targeting of the siRNA (Landen *et al.*, 2006; Morris and Rossi, 2006a; Oliveira *et al.*, 2006; Song *et al.*, 2005; Veldhoen *et al.*, 2006).

One unique approach to the problem of siRNA delivery is the use of lentiviral-mediated delivery of siRNA-expressing vectors into stem cells (Anderson *et al.*, 2006; Li *et al.*, 2005; Morris and Rossi, 2006a,b). Integration of an siRNA-expressing vector into a cell insures a stable and long-term suppression of a target gene. Combined with the ability of hematopoietic stem cells to develop into all of the HIV-1 target cells, it now becomes possible to engineer T cells and macrophages that carry specific siRNA. These vectors could be designed to target HIV-1 viral RNA, or downregulate expression of the appropriate coreceptor, thus preventing infection. These approaches may prove especially useful when applied to research in animals, especially the newly developed mouse model. In this new small animal model for HIV-1 infection, immune-deficient mice (Rag and common γ-chain knockout) are irradiated and then implanted with human $CD34^+$ hematopoietic stem cells (Baenziger *et al.*, 2006; Berges *et al.*, 2006; Watanabe *et al.*, 2006; Zhang *et al.*, 2006). Our laboratory has shown that these mice develop a human immune system, are capable of being infected with HIV-1, and have further shown the validity of using this model in evaluating drugs and small peptide therapeutics (unpublished data). This model could also be adapted to use stem cells carrying an anti-HIV siRNA or other siRNA of interest and be used to screen potential drugs and viral–cellular interactions.

The development of siRNA libraries allows for the rapid identification of genes important to HIV-1 infection (Nguyen *et al.*, 2006). Treatment of HIV-1 infected cells with a library of siRNA covering all known human genes, followed by screening for viral production, allows genes that influence HIV-1 infection to be identified. Works by several groups have shown these studies to be effective and have identified several genes involved in viral replication, including the group I PAK kinases; PAK1, PAK2, and PAK3 (Nguyen *et al.*, 2006). This provides a system wherein viral contact with cellular pathways can be identified without screening for interactions. Additionally, this approach allows the identification of genes that influence the virus but are not themselves differentially regulated by the virus.

VII. Conclusion

The disciplines of genomics and proteomics afford us a new way of examining HIV-1 infection, its interaction with cellular proteins, the changes the virus induces in the host cell, and the outcome of the course of the disease. By using a systems approach, in which information from

genomic, proteomic, and phenotypic studies is combined, new and more effective drug targets can be elucidated. Although extensive work has gone into identifying the interactions of viral proteins with the host cell, we are only now beginning to understand what these interactions entail and how they relate to specific stages of the viral life cycle. The work thus far performed on Tat, Nef, and Gag outlines how to apply an "omics" approach to understanding viral changes in the host cell network and how to counteract these changes using therapeutics. Future work involving rigorous systems biology approaches will undoubtedly uncover new targets for treatment of HIV/AIDS.

Acknowledgments

We would like to thank Reem Berro and Sergei Nekhai for critical review of this manuscript. This chapter explores a deep and very complex area of HIV research in a finite amount of space. As such, we would like to acknowledge our many colleagues in the field whose important work has advanced our understanding of the systems biology of HIV, but which we did not directly cite.

References

Agbottah, E., de La Fuente, C., Nekhai, S., Barnett, A., Gianella-Borradori, A., Pumfery, A., and Kashanchi, F. (2005). Antiviral activity of CYC202 in HIV-1-infected cells. *J. Biol. Chem.* **280**(4), 3029–3042.

Agbottah, E., Deng, L., Dannenberg, L. O., Pumfery, A., and Kashanchi, F. (2006). Effect of SWI/SNF chromatin remodeling complex on HIV-1 Tat activated transcription. *Retrovirology* **3**, 48.

Agostini, I., Navarro, J. M., Rey, F., Bouhamdan, M., Spire, B., Vigne, R., and Sire, J. (1996). The human immunodeficiency virus type 1 Vpr transactivator: Cooperation with promoter-bound activator domains and binding to TFIIB. *J. Mol. Biol.* **261**(5), 599–606.

Agrawal, N., Dasaradhi, P. V., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA interference: Biology, mechanism, and applications. *Microbiol. Mol. Biol. Rev.* **67**(4), 657–685.

Ammosova, T., Washington, K., Debebe, Z., Brady, J., and Nekhai, S. (2005). Dephosphorylation of CDK9 by protein phosphatase 2A and protein phosphatase-1 in Tat-activated HIV-1 transcription. *Retrovirology* **2**, 47.

Ammosova, T., Berro, R., Jerebtsova, M., Jackson, A., Charles, S., Klase, Z., Southerland, W., Gordeuk, V. R., Kashanchi, F., and Nekhai, S. (2006). Phosphorylation of HIV-1 Tat by CDK2 in HIV-1 transcription. *Retrovirology* **3**, 78.

Anderson, J. S., Bandi, S., Kaufman, D. S., and Akkina, R. (2006). Derivation of normal macrophages from human embryonic stem (hES) cells for applications in HIV gene therapy. *Retrovirology* **3**, 24.

Andersson, L. M., Hagberg, L., Rosengren, L., Fuchs, D., Blennow, K., and Gisslen, M. (2006). Normalisation of cerebrospinal fluid biomarkers parallels improvement of neurological symptoms following HAART in HIV dementia—case report. *BMC Infect. Dis.* **6**, 141.

Arold, S. T., and Baur, A. S. (2001). Dynamic Nef and Nef dynamics: How structure could explain the complex activities of this small HIV protein. *Trends Biochem. Sci.* **26**(6), 356–363.

Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M. O., Behnke, S., Frey, J., Oxenius, A., Joller, H., Aguzzi, A., Manz, M. G., *et al.* (2006). Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2−/− gamma c−/− mice. *Proc. Natl. Acad. Sci. USA* **103**(43), 15951–15956.

Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R., and Wortis, H. H. (2000). Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. *J. Exp. Med.* **191**(10), 1735–1744.

Bannwarth, S., and Gatignol, A. (2005). HIV-1 TAR RNA: The target of molecular interactions between the virus and its host. *Curr. HIV Res.* **3**(1), 61–71.

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116**(2), 281–297.

Benkirane, M., Chun, R. F., Xiao, H., Ogryzko, V. V., Howard, B. H., Nakatani, Y., and Jeang, K. T. (1998). Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem.* **273**(38), 24898–24905.

Benson, R. E., Sanfridson, A., Ottinger, J. S., Doyle, C., and Cullen, B. R. (1993). Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.* **177**(6), 1561–1566.

Bentham, M., Mazaleyrat, S., and Harris, M. (2006). Role of myristoylation and N-terminal basic residues in membrane association of the human immunodeficiency virus type 1 Nef protein. *J. Gen. Virol.* **87**(Pt 3), 563–571.

Berges, B. K., Wheat, W. H., Palmer, B. E., Connick, E., and Akkina, R. (2006). HIV-1 infection and CD4 T cell depletion in the humanized Rag2−/− gamma c−/− (RAG-hu) mouse model. *Retrovirology* **3**, 76.

Berkhout, B., and Jeang, K. T. (1989). Trans activation of human immunodeficiency virus type 1 is sequence specific for both the single-stranded bulge and loop of the trans-acting-responsive hairpin: A quantitative analysis. *J. Virol.* **63**(12), 5501–5504.

Berro, R., de la Fuente, C., Klase, Z., Kehn, K., Parvin, L., Pumfery, A., Agbottah, E., Vertes, A., Nekhai, S., and Kashanchi, F. (2007). Identify the membrane proteome of HIV-1 latently infected cells. *J. Biol. Chem.* **282**(11), 8207–8218.

Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1999). Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both necessary and sufficient for full activation of transcription. *Proc. Natl. Acad. Sci. USA* **96**(14), 7791–7796.

Blau, J., Xiao, H., McCracken, S., O'Hare, P., Greenblatt, J., and Bentley, D. (1996). Three functional classes of transcriptional activation domain. *Mol. Cell Biol.* **16**(5), 2044–2055.

Bohan, C. A., Kashanchi, F., Ensoli, B., Buonaguro, L., Boris-Lawrie, K. A., and Brady, J. N. (1992). Analysis of Tat transactivation of human immunodeficiency virus transcription *in vitro*. *Gene Expr.* **2**(4), 391–407.

Bouhamdan, M., Benichou, S., Rey, F., Navarro, J. M., Agostini, I., Spire, B., Camonis, J., Slupphaug, G., Vigne, R., Benarous, R., and Sire, J. (1996). Human immunodeficiency virus type 1 Vpr protein binds to the uracil DNA glycosylase DNA repair enzyme. *J. Virol.* **70**(2), 697–704.

Bour, S., Perrin, C., and Strebel, K. (1999). Cell surface CD4 inhibits HIV-1 particle release by interfering with Vpu activity. *J. Biol. Chem.* **274**(47), 33800–33806.

Brady, J., and Kashanchi, F. (2005). Tat gets the "green" light on transcription initiation. *Retrovirology* **2**, 69.

Bres, V., Kiernan, R., Emiliani, S., and Benkirane, M. (2002). Tat acetyl-acceptor lysines are important for human immunodeficiency virus type-1 replication. *J. Biol. Chem.* **277**(25), 22215–22221.

Bryant, M., and Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87**(2), 523–527.

Bukrinskaya, A., Brichacek, B., Mann, A., and Stevenson, M. (1998). Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. *J. Exp. Med.* **188**(11), 2113–2125.

Callahan, M. A., Handley, M. A., Lee, Y. H., Talbot, K. J., Harper, J. W., and Panganiban, A. T. (1998). Functional interaction of human immunodeficiency virus type 1 Vpu and Gag with a novel member of the tetratricopeptide repeat protein family. *J. Virol.* **72**(10), 8461.

Casella, C. R., Rapaport, E. L., and Finkel, T. H. (1999). Vpu increases susceptibility of human immunodeficiency virus type 1-infected cells to fas killing. *J. Virol.* **73**(1), 92–100.

Chang, L. J., Liu, X., and He, J. (2005). Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1. *Gene Ther.* **12**(14), 1133–1144.

Chao, S. H., Fujinaga, K., Marion, J. E., Taube, R., Sausville, E. A., Senderowicz, A. M., Peterlin, B. M., and Price, D. H. (2000). Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. *J. Biol. Chem.* **275**(37), 28345–28348.

Chauhan, A., Turchan, J., Pocernich, C., Bruce-Keller, A., Roth, S., Butterfield, D. A., Major, E. O., and Nath, A. (2003). Intracellular human immunodeficiency virus Tat expression in astrocytes promotes astrocyte survival but induces potent neurotoxicity at distant sites via axonal transport. *J. Biol. Chem.* **278**(15), 13512–13519.

Chen, D., Fong, Y., and Zhou, Q. (1999). Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription. *Proc. Natl. Acad. Sci. USA* **96**(6), 2728–2733.

Chen, R., Yang, Z., and Zhou, Q. (2004). Phosphorylated positive transcription elongation factor b (P-TEFb) is tagged for inhibition through association with 7SK snRNA. *J. Biol. Chem.* **279**(6), 4153–4160.

Chertova, E., Chertov, O., Coren, L. V., Roser, J. D., Trubey, C. M., Bess, J. W., Jr., Sowder, R. C., 2nd, Barsov, E., Hood, B. L., Fisher, R. J., Nagashima, K., Conrads, T. P., *et al.* (2006). Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. *J. Virol.* **80**(18), 9039–9052.

Chiang, C. M., and Roeder, R. G. (1995). Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators. *Science* **267**(5197), 531–536.

Chun, R. F., Semmes, O. J., Neuveut, C., and Jeang, K. T. (1998). Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. *J. Virol.* **72**(4), 2615–2629.

Ciborowski, P., and Gendelman, H. E. (2006). Human immunodeficiency virus-mononuclear phagocyte interactions: Emerging avenues of biomarker discovery, modes of viral persistence and disease pathogenesis. *Curr. HIV Res.* **4**(3), 279–291.

Cimarelli, A., and Luban, J. (1999). Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. *J. Virol.* **73**(7), 5388–5401.

Clark, E., Santiago, F., Deng, L., Chong, S., de La Fuente, C., Wang, L., Fu, P., Stein, D., Denny, T., Lanka, V., Mozafari, F., Okamoto, T., *et al.* (2000). Loss of G(1)/S checkpoint in human immunodeficiency virus type 1-infected cells is associated with a lack of cyclin-dependent kinase inhibitor p21/Waf1. *J. Virol.* **74**(11), 5040–5052.

Coffin, J. M., Hughes, S. H., and Varmus, H. E. (1997). "Retroviruses." Cold Spring Harbor Laboratory Press, Plainview, NY.

Cohrs, R. J., Gilden, D. H., Gomi, Y., Yamanishi, K., and Cohen, J. I. (2006). Comparison of virus transcription during lytic infection of the Oka parental and vaccine strains of Varicella-Zoster virus. *J. Virol.* **80**(5), 2076–2082.

Coiras, M., Camafeita, E., Urena, T., Lopez, J. A., Caballero, F., Fernandez, B., Lopez-Huertas, M. R., Perez-Olmeda, M., and Alcami, J. (2006). Modifications in the human T cell proteome induced by intracellular HIV-1 Tat protein expression. *Proteomics* **6**(Suppl. 1), S63–S73.

Col, E., Caron, C., Seigneurin-Berny, D., Gracia, J., Favier, A., and Khochbin, S. (2001). The histone acetyltransferase, hGCN5, interacts with and acetylates the HIV transactivator, Tat. *J. Biol. Chem.* **276**(30), 28179–28184.

Colbere-Garapin, F., Blondel, B., Saulnier, A., Pelletier, I., and Labadie, K. (2005). Silencing viruses by RNA interference. *Microbes Infect.* **7**(4), 767–775.

Coleman, S. H., Hitchin, D., Noviello, C. M., and Guatelli, J. C. (2006). HIV-1 Nef stabilizes AP-1 on membranes without inducing ARF1-independent de novo attachment. *Virology* **345**(1), 148–155.

Conejero-Goldberg, C., Wang, E., Yi, C., Goldberg, T. E., Jones-Brando, L., Marincola, F. M., Webster, M. J., and Torrey, E. F. (2005). Infectious pathogen detection arrays: Viral detection in cell lines and postmortem brain tissue. *Biotechniques* **39**(5), 741–751.

Cordingley, M. G., LaFemina, R. L., Callahan, P. L., Condra, J. H., Sardana, V. V., Graham, D. J., Nguyen, T. M., LeGrow, K., Gotlib, L., and Schlabach, A. J. (1990). Sequence-specific interaction of Tat protein and Tat peptides with the transactivation-responsive sequence element of human immunodeficiency virus type 1 *in vitro*. *Proc. Natl. Acad. Sci. USA* **87**(22), 8985–8989.

Cortes, M. J., Wong-Staal, F., and Lama, J. (2002). Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells. *J. Biol. Chem.* **277**(3), 1770–1779.

Craig, H. M., Reddy, T. R., Riggs, N. L., Dao, P. P., and Guatelli, J. C. (2000). Interactions of HIV-1 nef with the mu subunits of adaptor protein complexes 1, 2, and 3: Role of the dileucine-based sorting motif. *Virology* **271**(1), 9–17.

Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. O., and Peterlin, B. M. (1997). The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. *Genes Dev.* **11**(20), 2645–2657.

Cullen, B. R. (1991). Regulation of HIV-1 gene expression. *FASEB J.* **5**(10), 2361–2368.

Dal Monte, P., Landini, M. P., Sinclair, J., Virelizier, J. L., and Michelson, S. (Monte 1997). TAR and Sp1-independent transactivation of HIV long terminal repeat by the Tat protein in the presence of human cytomegalovirus IE1/IE2. *AIDS* **11**(3), 297–303.

Das, S. R., and Jamel, S. (2005). Biology of the HIV Nef protein. *Indian J. Med. Res.* **121**, 315–332.

Daube, H., Billich, A., Mann, K., and Schramm, H. J. (1991). Cleavage of phosphorylase kinase and calcium-free calmodulin by HIV-1 protease. *Biochem. Biophys. Res. Commun.* **178**(3), 892–898.

de Falco, G., and Giordano, A. (Falco 1998). CDK9 (PITALRE): A multifunctional cdc2-related kinase. *J. Cell Physiol.* **177**(4), 501–506.

de la Fuente, C., Santiago, F., Deng, L., Eadie, C., Zilberman, I., Kehn, K., Maddukuri, A., Baylor, S., Wu, K., Lee, C. G., Pumfery, A., and Kashanchi, F. (la Fuente 2002). Gene expression profile of HIV-1 Tat expressing cells: A close interplay between proliferative and differentiation signals. *BMC Biochem.* **3**, 14.

de la Fuente, C., Maddukuri, A., Kehn, K., Baylor, S. Y., Deng, L., Pumfery, A., and Kashanchi, F. (2003). Pharmacological cyclin-dependent kinase inhibitors as HIV-1 antiviral therapeutics. *Curr. HIV Res.* **1**(2), 131–152.

Deng, L., de la Fuente, C., Fu, P., Wang, L., Donnelly, R., Wade, J. D., Lambert, P., Li, H., Lee, C. G., and Kashanchi, F. (2000). Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones. *Virology* **277**(2), 278–295.

Deng, L., Wang, D., de la Fuente, C., Wang, L., Li, H., Lee, C. G., Donnelly, R., Wade, J. D., Lambert, P., and Kashanchi, F. (2001). Enhancement of the p300 HAT activity by HIV-1 Tat on chromatin DNA. *Virology* **289**(2), 312–326.

Deng, L., Ammosova, T., Pumfery, A., Kashanchi, F., and Nekhai, S. (2002). HIV-1 Tat interaction with RNA polymerase II C-terminal domain (CTD) and a dynamic association

with CDK2 induce CTD phosphorylation and transcription from HIV-1 promoter. *J. Biol. Chem.* **277**(37), 33922–33929.

Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Heaphy, S., Karn, J., Lowe, A. D., Singh, M., and Skinner, M. A. (1990). HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. *EMBO J.* **9**(12), 4145–4153.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**(6836), 494–498.

Endrich, M. M., Gehrig, P., and Gehring, H. (1999). Maturation-induced conformational changes of HIV-1 capsid protein and identification of two high affinity sites for cyclophilins in the C-terminal domain. *J. Biol. Chem.* **274**(9), 5326–5332.

Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R. A., Wingfield, P., and Gallo, R. C. (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* **67**(1), 277–287.

Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P., and Laemmli, U. K. (1991). Specific complex of human immunodeficiency virus type 1 rev and nucleolar B23 proteins: Dissociation by the Rev response element. *Mol. Cell Biol.* **11**(5), 2567–2575.

Farjot, G., Sergeant, A., and Mikaelian, I. (1999). A new nucleoporin-like protein interacts with both HIV-1 Rev nuclear export signal and CRM-1. *J. Biol. Chem.* **274**(24), 17309–17317.

Faulkner, N. E., Lane, B. R., Bock, P. J., and Markovitz, D. M. (2003). Protein phosphatase 2A enhances activation of human immunodeficiency virus type 1 by phorbol myristate acetate. *J. Virol.* **77**(3), 2276–2281.

Feinberg, M. B., Baltimore, D., and Frankel, A. D. (1991). The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. USA* **88**(9), 4045–4049.

Felzien, L. K., Woffendin, C., Hottiger, M. O., Subbramanian, R. A., Cohen, E. A., and Nabel, G. J. (1998). HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 co-activator. *Proc. Natl. Acad. Sci. USA* **95**(9), 5281–5286.

Feng, S., and Holland, E. C. (1988). HIV-1 tat trans-activation requires the loop sequence within tar. *Nature* **334**(6178), 165–167.

Fiorentini, S., Marini, E., Caracciolo, S., and Caruso, A. (2006). Functions of the HIV-1 matrix protein p17. *New Microbiol.* **29**(1), 1–10.

Fong, Y. W., and Zhou, Q. (2000). Relief of two built-In autoinhibitory mechanisms in P-TEFb is required for assembly of a multicomponent transcription elongation complex at the human immunodeficiency virus type 1 promoter. *Mol. Cell Biol.* **20**(16), 5897–5907.

Fraldi, A., Varrone, F., Napolitano, G., Michels, A. A., Majello, B., Bensaude, O., and Lania, L. (2005). Inhibition of Tat activity by the HEXIM1 protein. *Retrovirology* **2**(1), 42.

Freed, E. O. (1998). HIV-1 gag proteins: Diverse functions in the virus life cycle. *Virology* **251**(1), 1–15.

Freed, E. O. (2002). Viral late domains. *J. Virol.* **76**(10), 4679–4687.

Freed, E. O. (2003). The HIV-TSG101 interface: Recent advances in a budding field. *Trends Microbiol.* **11**(2), 56–59.

Fridell, R. A., Harding, L. S., Bogerd, H. P., and Cullen, B. R. (1995). Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins. *Virology* **209**(2), 347–357.

Fujinaga, K., Cujec, T. P., Peng, J., Garriga, J., Price, D. H., Grana, X., and Peterlin, B. M. (1998). The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. *J. Virol.* **72**(9), 7154–7159.

Fujinaga, K., Taube, R., Wimmer, J., Cujec, T. P., and Peterlin, B. M. (1999). Interactions between human cyclin T, Tat, and the transactivation response element (TAR) are

disrupted by a cysteine to tyrosine substitution found in mouse cyclin T. *Proc. Natl. Acad. Sci. USA* **96**(4), 1285–1290.

Fukumori, T., Kagawa, S., Iida, S., Oshima, Y., Akari, H., Koyama, A. H., and Adachi, A. (1999). Rev-dependent expression of three species of HIV-1 mRNAs (review). *Int. J. Mol. Med.* **3**(3), 297–302.

Gallay, P., Hope, T., Chin, D., and Trono, D. (1997). HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA* **94**(18), 9825–9830.

Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E., and Jones, K. A. (2000). CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol. Cell Biol.* **20** (18), 6958–6969.

Garcia, J. A., Harrich, D., Soultanakis, E., Wu, F., Mitsuyasu, R., and Gaynor, R. B. (1989). Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. *EMBO J.* **8**(3), 765–778.

Garcia-Martinez, L. F., Ivanov, D., and Gaynor, R. B. (1997). Association of Tat with purified HIV-1 and HIV-2 transcription preinitiation complexes. *J. Biol. Chem.* **272**(11), 6951–6958.

Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* **107**(1), 55–65.

Geleziunas, R., Xu, W., Takeda, K., Ichijo, H., and Greene, W. C. (2001). HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* **410**(6830), 834–838.

Geyer, M., Fackler, O. T., and Peterlin, B. M. (2001). Structure–function relationships in HIV-1 Nef. *EMBO Rep.* **2**(7), 580–585.

Ghedin, E., Pumfery, A., de la Fuente, C., Yao, K., Miller, N., Lacoste, V., Quackenbush, J., Jacobson, S., and Kashanchi, F. (2004). Use of a multi-virus array for the study of human viral and retroviral pathogens: Gene expression studies and ChIP-chip analysis. *Retrovirology* **1**(1), 10.

Goila-Gaur, R., Demirov, D. G., Orenstein, J. M., Ono, A., and Freed, E. O. (2003). Defects in human immunodeficiency virus budding and endosomal sorting induced by TSG101 overexpression. *J. Virol.* **77**(11), 6507–6519.

Gottlinger, H. G. (2001). The HIV-1 assembly machine. *AIDS* **15**(Suppl. 5), S13–S20.

Greenberg, M. E., Bronson, S., Lock, M., Neumann, M., Pavlakis, G. N., and Skowronski, J. (1997). Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. *EMBO J.* **16**(23), 6964–6976.

Greene, W. C., and Peterlin, B. M. (2002). Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. *Nat. Med.* **8**(7), 673–680.

Greenway, A. L., McPhee, D. A., Allen, K., Johnstone, R., Holloway, G., Mills, J., Azad, A., Sankovich, S., and Lambert, P. (2002). Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis. *J. Virol.* **76**(6), 2692–2702.

Grzesiek, S., Stahl, S. J., Wingfield, P. T., and Bax, A. (1996). The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry* **35**(32), 10256–10261.

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., and Dandekar, S. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**(21), 11708–11717.

Hannon, G. J. (2002). RNA interference. *Nature* **418**(6894), 244–251.

Harris, M. (1995). The role of myristoylation in the interactions between human immunodeficiency virus type I Nef and cellular proteins. *Biochem. Soc. Trans.* **23**(3), 557–561.

Herrmann, C. H., and Mancini, M. A. (2001). The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. *J. Cell Sci.* **114**(Pt 8), 1491–1503.

Hope, T., and Pomerantz, R. J. (1995). The human immunodeficiency virus type 1 Rev protein: A pivotal protein in the viral life cycle. *Curr. Top. Microbiol. Immunol.* **193**, 91–105.

Hope, T. J. (1997). Viral RNA export. *Chem. Biol.* **4**(5), 335–344.

Hottiger, M. O., and Nabel, G. J. (1998). Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. *J. Virol.* **72**(10), 8252–8256.

Islam, T. C., and Smith, C. I. (2000). The cellular phenotype conditions Btk for cell survival or apoptosis signaling. *Immunol. Rev.* **178**, 49–63.

Izmailova, E., Bertley, F. M., Huang, Q., Makori, N., Miller, C. J., Young, R. A., and Aldovini, A. (2003). HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages. *Nat. Med.* **9**(2), 191–197.

Jacotot, E., Ferri, K. F., El Hamel, C., Brenner, C., Druillennec, S., Hoebeke, J., Rustin, P., Metivier, D., Lenoir, C., Geuskens, M., Vieira, H. L., Loeffler, M., *et al.* (2001). Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2. *J. Exp. Med.* **193**(4), 509–519.

Jacque, J. M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418**(6896), 435–438.

Janardhan, A., Swigut, T., Hill, B., Myers, M. P., and Skowronski, J. (2004). HIV-1 Nef binds the DOCK2-ELMO1 complex to activate rac and inhibit lymphocyte chemotaxis. *PLoS Biol.* **2**(1), E6.

Janvier, K., Craig, H., Le Gall, S., Benarous, R., Guatelli, J., Schwartz, O., and Benichou, S. (2001). Nef-induced CD4 downregulation: A diacidic sequence in human immunodeficiency virus type 1 Nef does not function as a protein sorting motif through direct binding to beta-COP. *J. Virol.* **75**(8), 3971–3976.

Jeang, K. T., Xiao, H., and Rich, E. A. (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J. Biol. Chem.* **274**(41), 28837–28840.

Kamine, J., Subramanian, T., and Chinnadurai, G. (1991). Sp1-dependent activation of a synthetic promoter by human immunodeficiency virus type 1 Tat protein. *Proc. Natl. Acad. Sci. USA* **88**(19), 8510–8514.

Kamine, J., Elangovan, B., Subramanian, T., Coleman, D., and Chinnadurai, G. (1996). Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator. *Virology* **216**(2), 357–366.

Karczewski, M. K., and Strebel, K. (1996). Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *J. Virol.* **70**(1), 494–507.

Karn, J. (1999). Tackling Tat. *J. Mol. Biol.* **293**(2), 235–254.

Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C. M., Roeder, R. G., and Brady, J. N. (1994). Direct interaction of human TFIID with the HIV-1 transactivator tat. *Nature* **367**(6460), 295–299.

Kashanchi, F., Khleif, S. N., Duvall, J. F., Sadaie, M. R., Radonovich, M. F., Cho, M., Martin, M. A., Chen, S. Y., Weinmann, R., and Brady, J. N. (1996). Interaction of human immunodeficiency virus type 1 Tat with a unique site of TFIID inhibits negative cofactor Dr1 and stabilizes the TFIID-TFIIA complex. *J. Virol.* **70**(8), 5503–5510.

Kato, H., Sumimoto, H., Pognonec, P., Chen, C. H., Rosen, C. A., and Roeder, R. G. (1992). HIV-1 Tat acts as a processivity factor *in vitro* in conjunction with cellular elongation factors. *Genes Dev.* **6**(4), 655–666.

Kellam, P. (2001). Post-genomic virology: The impact of bioinformatics, microarrays, and proteomics on investigating host and pathogen interactions. *Rev. Med. Virol.* **11**(5), 313–329.

Kiernan, R. E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomme, C., Burny, A., Nakatani, Y., Jeang, K. T., Benkirane, M., and Van Lint, C. (1999). HIV-1 tat transcriptional activity is regulated by acetylation. *EMBO J.* **18**(21), 6106–6118.

Kim, Y. K., Bourgeois, C. F., Isel, C., Churcher, M. J., and Karn, J. (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. *Mol. Cell Biol.* **22**(13), 4622–4637.

Kostrzynska, M., and Bachand, A. (2006). Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens. *Can. J. Microbiol.* **52**(1), 1–8.

Landen, C. N., Merritt, W. M., Mangala, L. S., Sanguino, A. M., Bucana, C., Lu, C., Lin, Y. G., Han, L. Y., Kamat, A. A., Schmandt, R., Coleman, R. L., Gershenson, D. M., *et al.* (2006). Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol. Ther.* **5**(12), 1708–1713.

Laspia, M. F., Rice, A. P., and Mathews, M. B. (1989). HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59**(2), 283–292.

Lee, C. H., Saksela, K., Mirza, U. A., Chait, B. T., and Kuriyan, J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell* **85**(6), 931–942.

Li, F., and Wild, C. (2005). HIV-1 assembly and budding as targets for drug discovery. *Curr. Opin. Investig. Drugs* **6**(2), 148–154.

Li, M. J., Kim, J., Li, S., Zaia, J., Yee, J. K., Anderson, J., Akkina, R., and Rossi, J. J. (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol. Ther.* **12**(5), 900–909.

Liang, W. S., Maddukuri, A., Teslovich, T. M., de la Fuente, C., Agbottah, E., Dadgar, S., Kehn, K., Hautaniemi, S., Pumfery, A., Stephan, D. A., and Kashanchi, F. (2005). Therapeutic targets for HIV-1 infection in the host proteome. *Retrovirology* **2**(1), 20.

Lin, C. W., and Engelman, A. (2003). The barrier-to-autointegration factor is a component of functional human immunodeficiency virus type 1 preintegration complexes. *J. Virol.* **77**(8), 5030–5036.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379**(6563), 349–353.

Liu, B., Dai, R., Tian, C. J., Dawson, L., Gorelick, R., and Yu, X. F. (1999). Interaction of the human immunodeficiency virus type 1 nucleocapsid with actin. *J. Virol.* **73**(4), 2901–2908.

Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R., and Benichou, S. (1997). Binding of HIV-1 Nef to a novel thioesterase enzyme correlates with Nef-mediated CD4 down-regulation. *J. Biol. Chem.* **272**(21), 13779–13785.

Lu, X., Yu, H., Liu, S. H., Brodsky, F. M., and Peterlin, B. M. (1998). Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. *Immunity* **8**(5), 647–656.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**(6), 1067–1078.

Madani, N., Millette, R., Platt, E. J., Marin, M., Kozak, S. L., Bloch, D. B., and Kabat, D. (2002). Implication of the lymphocyte-specific nuclear body protein Sp140 in an innate response to human immunodeficiency virus type 1. *J Virol.* **76**(21), 11133–11138.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G. D., Muschel, R. J., and Weiner, D. B. (1998). HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a

cellular factor linked to the G2/M phase transition of the mammalian cell cycle. *Proc. Natl. Acad. Sci. USA* **95**(7), 3419–3424.

Mahmoudi, T., Parra, M., Vries, R. G., Kauder, S. E., Verrijzer, C. P., Ott, M., and Verdin, E. (2006). The SWI/SNF chromatin-remodeling complex is a cofactor for Tat transactivation of the HIV promoter. *J. Biol. Chem.* **281**(29), 19960–19968.

Majello, B., Napolitano, G., Giordano, A., and Lania, L. (1999). Transcriptional regulation by targeted recruitment of cyclin-dependent CDK9 kinase *in vivo*. *Oncogene* **18**(32), 4598–4605.

Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpentier, J. L., and Trono, D. (1997). The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. *Immunity* **6**(1), 67–77.

Mansharamani, M., Graham, D. R., Monie, D., Lee, K. K., Hildreth, J. E., Siliciano, R. F., and Wilson, K. L. (2003). Barrier-to-autointegration factor BAF binds p55 Gag and matrix and is a host component of human immunodeficiency virus type 1 virions. *J. Virol.* **77**(24), 13084–13092.

Marciniak, R. A., and Sharp, P. A. (1991). HIV-1 Tat protein promotes formation of more-processive elongation complexes. *EMBO J.* **10**(13), 4189–4196.

Marciniak, R. A., Calnan, B. J., Frankel, A. D., and Sharp, P. A. (1990). HIV-1 Tat protein trans-activates transcription *in vitro*. *Cell* **63**(4), 791–802.

Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998). A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Mol. Cell* **1**(4), 565–574.

McDougal, J. S., Kennedy, M. S., Sligh, J. M., Cort, S. P., Mawle, A., and Nicholson, J. K. (1986). Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. *Science* **231**(4736), 382–385.

Michels, A. A., Fraldi, A., Li, Q., Adamson, T. E., Bonnet, F., Nguyen, V. T., Sedore, S. C., Price, J. P., Price, D. H., Lania, L., and Bensaude, O. (2004). Binding of the 7SK snRNA turns the HEXIM1 protein into a P-TEFb (CDK9/cyclin T) inhibitor. *EMBO J.* **23**(13), 2608–2619.

Misumi, S., Fuchigami, T., Takamune, N., Takahashi, I., Takama, M., and Shoji, S. (2002). Three isoforms of cyclophilin A associated with human immunodeficiency virus type 1 were found by proteomics by using two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Virol.* **76**(19), 10000–10008.

Mohrmann, L., Langenberg, K., Krijgsveld, J., Kal, A. J., Heck, A. J., and Verrijzer, C. P. (2004). Differential targeting of two distinct SWI/SNF-related Drosophila chromatin-remodeling complexes. *Mol. Cell Biol.* **24**(8), 3077–3088.

Morris, K. V., and Rossi, J. J. (2006a). Lentiviral-mediated delivery of siRNAs for antiviral therapy. *Gene Ther.* **13**(6), 553–558.

Morris, K. V., and Rossi, J. J. (2006b). Lentivirus-mediated RNA interference therapy for human immunodeficiency virus type 1 infection. *Hum. Gene Ther.* **17**, 479.

Mujtaba, S., He, Y., Zeng, L., Farooq, A., Carlson, J. E., Ott, M., Verdin, E., and Zhou, M. M. (2002). Structural basis of lysine-acetylated HIV-1 Tat recognition by PCAF bromodomain. *Mol. Cell* **9**(3), 575–586.

Nelson, P. J., D'Agati, V. D., Gries, J. M., Suarez, J. R., and Gelman, I. H. (2003). Amelioration of nephropathy in mice expressing HIV-1 genes by the cyclin-dependent kinase inhibitor flavopiridol. *J. Antimicrob. Chemother.* **51**(4), 921–929.

Neville, M., Stutz, F., Lee, L., Davis, L. I., and Rosbash, M. (1997). The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.* **7**(10), 767–775.

New, D. R., Ma, M., Epstein, L. G., Nath, A., and Gelbard, H. A. (1997). Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. *J. Neurovirol.* **3**(2), 168–173.

Nguyen, D. G., Wolff, K. C., Yin, H., Caldwell, J. S., and Kuhen, K. L. (2006). "UnPAKing" human immunodeficiency virus (HIV) replication: Using small interfering RNA screening to identify novel cofactors and elucidate the role of group I PAKs in HIV infection. *J. Virol.* **80**(1), 130–137.

O'Keeffe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000). Requirement for a kinase-specific chaperone pathway in the production of a Cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated tat stimulation of HIV-1 transcription. *J. Biol. Chem.* **275**(1), 279–287.

Oliveira, S., Storm, G., and Schiffelers, R. M. (2006). Targeted delivery of siRNA. *J. Biomed. Biotechnol.* **2006**(4), 63675.

O'Neill, E., Kuo, L. S., Krisko, J. F., Tomchick, D. R., Garcia, J. V., and Foster, J. L. (2006). Dynamic evolution of the human immunodeficiency virus type 1 pathogenic factor, Nef. *J. Virol.* **80**(3), 1311–1320.

Ott, M., Schnolzer, M., Garnica, J., Fischle, W., Emiliani, S., Rackwitz, H. R., and Verdin, E. (1999). Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. *Curr. Biol.* **9**(24), 1489–1492.

Parada, C. A., and Roeder, R. G. (1996). Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain. *Nature* **384**(6607), 375–378.

Paroli, M., Propato, A., Accapezzato, D., Francavilla, V., Schiaffella, E., and Barnaba, V. (2001). The immunology of HIV-infected long-term non-progressors—a current view. *Immunol. Lett.* **79**(1–2), 127–129.

Peterlin, B. M., and Trono, T. (2003). Hide, shield, and strike back: How HIV-infected cells avoid immune eradication. *Nat. Rev. Immunol.* **3**(2), 97–107.

Petro, J. B., Rahman, S. M., Ballard, D. W., and Khan, W. N. (2000). Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *J. Exp. Med.* **191**(10), 1745–1754.

Petro, J. B., Castro, I., Lowe, J., and Khan, W. N. (2002). Bruton's tyrosine kinase targets NF-kappaB to the bcl-x promoter via a mechanism involving phospholipase C-gamma2 following B cell antigen receptor engagement. *FEBS Lett.* **532**(1–2), 57–60.

Peytavi, R., Hong, S. S., Gay, B., d'Angeac, A. D., Selig, L., Benichou, S., Benarous, R., and Boulanger, P. (1999). HEED, the product of the human homolog of the murine *eed* gene, binds to the matrix protein of HIV-1. *J. Biol. Chem.* **274**(3), 1635–1645.

Piguet, V., Gu, F., Foti, M., Demaurex, N., Gruenberg, J., Carpentier, J. L., and Trono, D. (1999). Nef-induced CD4 degradation: A diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes. *Cell* **97**(1), 63–73.

Piguet, V., Wan, L., Borel, C., Mangasarian, A., Demaurex, N., Thomas, G., and Trono, D. (2000). HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. *Nat. Cell Biol.* **2**(3), 163–167.

Pocernich, C. B., Boyd-Kimball, D., Poon, H. F., Thongboonkerd, V., Lynn, B. C., Klein, J. B., Calebrese, V., Nath, A., and Butterfield, D. A. (2005). Proteomics analysis of human astrocytes expressing the HIV protein Tat. *Brain Res. Mol. Brain Res.* **133**(2), 307–316.

Provost, P., Barat, C., Plante, I., and Tremblay, M. J. (2006). HIV-l and the microRNA-guided silencing pathway: An intricate and multifaceted encounter. *Virus Res.* **121**(2), 117–115.

Radding, W., Williams, J. P., McKenna, M. A., Tummala, R., Hunter, E., Tytler, E. M., and McDonald, J. M. (2000). Calmodulin and HIV type 1: Interactions with Gag and Gag products. *AIDS Res. Hum. Retroviruses* **16**(15), 1519–1525.

Raha, T., Cheng, S. W., and Green, M. R. (2005). HIV-1 Tat stimulates transcription complex assembly through recruitment of TBP in the absence of TAFs. *PLoS Biol.* **3**(2), e44.

Ramanathan, Y., Reza, S. M., Young, T. M., Mathews, M. B., and Pe'ery, T. (1999). Human and rodent transcription elongation factor P-TEFb: Interactions with human immunodeficiency virus type 1 tat and carboxy-terminal domain substrate. *J. Virol.* **73**(7), 5448–5458.

Reeves, J. D., and Piefer, A. J. (2005). Emerging drug targets for antiretroviral therapy. *Drugs* **65**(13), 1747–1766.

Richter, S., Ping, Y. H., and Rana, T. M. (2002). TAR RNA loop: A scaffold for the assembly of a regulatory switch in HIV replication. *Proc. Natl. Acad. Sci. USA* **99**(12), 7928–7933.

Roebuck, K. A., Rabbi, M. F., and Kagnoff, M. F. (1997). HIV-1 Tat protein can transactivate a heterologous TATAA element independent of viral promoter sequences and the transactivation response element. *AIDS* **11**(2), 139–146.

Roeth, J. F., and Collins, K. L. (2006). Human immunodeficiency virus type 1 Nef: Adapting to intracellular trafficking pathways. *Microbiol. Mol. Biol. Rev.* **70**(2), 548–563.

Roeth, J. F., Williams, M., Kasper, M. R., Filzen, T. M., and Collins, K. L. (2004). HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail. *J. Cell Biol.* **167**(5), 903–913.

Roy, S., Delling, U., Chen, C. H., Rosen, C. A., and Sonenberg, N. (1990). A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated trans-activation. *Genes Dev.* **4**(8), 1365–1373.

Ruhl, M., Himmelspach, M., Bahr, G. M., Hammerschmid, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G. K., Probst, H., and Bevec, D. (1993). Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. *J. Cell Biol.* **123**(6 Pt 1), 1309–1320.

Ryabinin, V. A., Shundrin, L. A., Kostina, E. B., Laassri, M., Chizhikov, V., Shchelkunov, S. N., Chumakov, K., and Sinyakov, A. N. (2006). Microarray assay for detection and discrimination of Orthopoxvirus species. *J. Med. Virol.* **78**(10), 1325–1340.

Sankaran, S., Guadalupe, M., Reay, E., George, M. D., Flamm, J., Prindiville, T., and Dandekar, S. (2005). Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. *Proc. Natl. Acad. Sci. USA* **102**(28), 9860–9865.

Saphire, A. C., Gallay, P. A., and Bark, S. J. (2006). Proteomic analysis of human immunodeficiency virus using liquid chromatography/tandem mass spectrometry effectively distinguishes specific incorporated host proteins. *J. Proteome Res.* **5**(3), 530–538.

Sawaya, B. E., Khalili, K., Mercer, W. E., Denisova, L., and Amini, S. (1998). Cooperative actions of HIV-1 Vpr and p53 modulate viral gene transcription. *J. Biol. Chem.* **273**(32), 20052–20057.

Sawaya, B. E., Khalili, K., Gordon, J., Taube, R., and Amini, S. (2000). Cooperative interaction between HIV-1 regulatory proteins Tat and Vpr modulates transcription of the viral genome. *J. Biol. Chem.* **275**(45), 35209–35214.

Schimmer, A. D., Welsh, K., Pinilla, C., Wang, Z., Krajewska, M., Bonneau, M. J., Pedersen, I. M., Kitada, S., Scott, F. L., Bailly-Maitre, B., Glinsky, G., Scudiero, D., *et al.* (2004). Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* **5**(1), 25–35.

Schulte, A., Czudnochowski, N., Barboric, M., Schonichen, A., Blazek, D., Peterlin, B. M., and Geyer, M. (2005). Identification of a cyclin T-binding domain in Hexim1 and biochemical analysis of its binding competition with HIV-1 Tat. *J. Biol. Chem.* **280**(26), 24968–24977.

Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F., and Heard, J. M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat. Med.* **2**(3), 338–342.

Sedlacek, H. H. (2001). Mechanisms of action of flavopiridol. *Crit. Rev. Oncol. Hematol.* **38**(2), 139–170.

Selby, M. J., Bain, E. S., Luciw, P. A., and Peterlin, B. M. (1989). Structure, sequence, and position of the stem-loop in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat. *Genes Dev.* **3**(4), 547–558.

Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat. Med.* **9**(11), 1404–1407.

Simmons, A., Aluvihare, V., and McMichael, A. (2001). Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* **14**(6), 763–777.

Song, E., Zhu, P., Lee, S. K., Chowdhury, D., Kussman, S., Dykxhoorn, D. M., Feng, Y., Palliser, D., Weiner, D. B., Shankar, P., Marasco, W. A., and Lieberman, J. (2005). Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**(6), 709–717.

Sorin, M., and Kalpana, G. V. (2006). Dynamics of virus-host interplay in HIV-1 replication. *Curr. HIV Res.* **4**(2), 117–130.

Strack, B., Calistri, A., Craig, S., Popova, E., and Gottlinger, H. G. (2003). AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* **114**(6), 689–699.

Suzuki, K., Shijuuku, T., Fukamachi, T., Zaunders, J., Guillemin, G., Cooper, D., and Kelleher, A. (2005). Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J. RNAi Gene Silencing* **1**(2), 66–78.

Swann, S. A., Williams, M., Story, C. M., Bobbitt, K. R., Fleis, R., and Collins, K. L. (2001). HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. *Virology* **282**, 267–277.

Tang, H., Peng, T., and Wong-Staal, F. (2002). Novel technologies for studying virus-host interaction and discovering new drug targets for HCV and HIV. *Curr. Opin. Pharmacol.* **2**(5), 541–547.

Tange, T. O., Jensen, T. H., and Kjems, J. (1996). *In vitro* interaction between human immunodeficiency virus type 1 Rev protein and splicing factor ASF/SF2-associated protein, p32. *J. Biol. Chem.* **271**(17), 10066–10072.

Tasara, T., Hottiger, M. O., and Hubscher, U. (2001). Functional genomics in HIV-1 virus replication: Protein-protein interactions as a basis for recruiting the host cell machinery for viral propagation. *Biol. Chem.* **382**(7), 993–999.

Taube, R., Lin, X., Irwin, D., Fujinaga, K., and Peterlin, B. M. (2002). Interaction between P-TEFb and the C-terminal domain of RNA polymerase II activates transcriptional elongation from sites upstream or downstream of target genes. *Mol. Cell Biol.* **22**(1), 321–331.

ter Brake, O., and Berkhout, B. (Brake 2005). A novel approach for inhibition of HIV-1 by RNA interference: Counteracting viral escape with a second generation of siRNAs. *J. RNAi Gene Silencing* **1**(2), 56–65.

Treand, C., du Chene, I., Bres, V., Kiernan, R., Benarous, R., Benkirane, M., and Emiliani, S. (2006). Requirementfor SWI/SNF chromatin-remodeling complex in Tat-mediated activation of the HIV-1 promoter. *EMBO J.* **25**(8), 1690–1609.

Truant, R., and Cullen, B. R. (1999). The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell Biol.* **19**(2), 1210–1217.

Valdez, H., Carlson, N. L., Post, A. B., Asaad, R., Heeger, P. S., Lederman, M. M., Lehmann, P. V., and Anthony, D. D. (2002). HIV long-term non-progressors maintain brisk CD8 T cell responses to other viral antigens. *AIDS* **16**(8), 1113–1118.

van't Wout, A. B., Swain, J. V., Schindler, M., Rao, U., Pathmajeyan, M. S., Mullins, J. I., and Kirchhoff, F. (Wout 2005). Nef induces multiple genes involved in cholesterol synthesis and uptake in human immunodeficiency virus type 1-infected T cells. *J. Virol.* **79**(15), 10053–10058.

Veldhoen, S., Laufer, S. D., Trampe, A., and Restle, T. (2006). Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: Quantitative analysis of uptake and biological effect. *Nucleic Acids Res.* **34**(19), 6561–6573.

VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J., and Carter, C. A. (2001). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc. Natl. Acad. Sci. USA* **98**(14), 7724–7729.

Veschambre, P., Simard, P., and Jalinot, P. (1995). Evidence for functional interaction between the HIV-1 Tat transactivator and the TATA box binding protein *in vivo*. *J. Mol. Biol.* **250**(2), 169–180.

Viscidi, R. P., Mayur, K., Lederman, H. M., and Frankel, A. D. (1989). Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. *Science* **246**(4937), 1606–1608.

von Schwedler, U. K., Stuchell, M., Muller, B., Ward, D. M., Chung, H. Y., Morita, E., Wang, H. E., Davis, T., He, G. P., Cimbora, D. M., Scott, A., Krausslich, H. G., *et al.* (Schwedler 2003). The protein network of HIV budding. *Cell* **114**(6), 701–713.

Wang, D., de la Fuente, C., Deng, L., Wang, L., Zilberman, I., Eadie, C., Healey, M., Stein, D., Denny, T., Harrison, L. E., Meijer, L., and Kashanchi, F. (2001). Inhibition of human immunodeficiency virus type 1 transcription by chemical cyclin-dependent kinase inhibitors. *J. Virol.* **75**(16), 7266–7279.

Watanabe, H., Shiratori, T., Shoji, H., Miyatake, S., Okazaki, Y., Ikuta, K., Sato, T., and Saito, T. (1997). A novel acyl-CoA thioesterase enhances its enzymatic activity by direct binding with HIV Nef. *Biochem. Biophys. Res. Commun.* **238**(1), 234–239.

Watanabe, S., Terashima, K., Ohta, S., Horibata, S., Yajima, M., Shiozawa, Y., Dewan, M. Z., Yu, Z., Ito, M., Morio, T., Shimizu, N., Honda, M., *et al.* (2006). Hematopoietic stem cell-engrafted NOD/SCID/IL2R{gamma}null mice develop human lymphoid system and induce long-lasting HIV-1 infection with specific humoral immune responses. *Blood* **109**(1), 212–218.

Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**(4), 451–462.

Westendorp, M. O., Li-Weber, M., Frank, R. W., and Krammer, P. H. (1994). Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *J. Virol.* **68**(7), 4177–4185.

Westerhout, E. M., Ooms, M., Vink, M., Das, A. T., and Berkhout, B. (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.* **33**(2), 796–804.

Willetts, K. E., Rey, F., Agostini, I., Navarro, J. M., Baudat, Y., Vigne, R., and Sire, J. (1999). DNA repair enzyme uracil DNA glycosylase is specifically incorporated into human immunodeficiency virus type 1 viral particles through a Vpr-independent mechanism. *J. Virol.* **73**(2), 1682–1688.

Williams, M., Roeth, J. F., Kasper, M. R., Fleis, R. I., Przybycin, C. G., and Collins, K. L. (2002). Direct binding of human immunodeficiency virus type 1 Nef to the major histocompatibility complex class I (MHC-I) cytoplasmic tail disrupts MHC-I trafficking. *J. Virol.* **76**(23), 12173–12184.

Wilson, S. A., Sieiro-Vazquez, C., Edwards, N. J., Iourin, O., Byles, E. D., Kotsopoulou, E., Adamson, C. S., Kingsman, S. M., Kingsman, A. J., and Martin-Rendon, E. (1999). Cloning and characterization of hIF2, a human homologue of bacterial translation initiation factor 2, and its interaction with HIV-1 matrix. *Biochem. J.* **342**(Pt 1), 97–103.

Winston, F. (2001). Control of eukaryotic transcription elongation. *Genome Biol.* **2**(2), reviews1006.1–reviews1006.3.

Withers-Ward, E. S., Jowett, J. B., Stewart, S. A., Xie, Y. M., Garfinkel, A., Shibagaki, Y., Chow, S. A., Shah, N., Hanaoka, F., Sawitz, D. G., Armstrong, R. W., Souza, L. M., *et al.*

(1997). Human immunodeficiency virus type 1 Vpr interacts with HHR23A, a cellular protein implicated in nucleotide excision DNA repair. *J. Virol.* **71**(12), 9732–9742.

Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d'Aloja, P., Schurmann, A., and Baur, A. S. (2001). HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. *Nat. Med.* **7**(11), 1217–1224.

Wu-Baer, F., Lane, W. S., and Gaynor, R. B. (1998). Role of the human homolog of the yeast transcription factor SPT5 in HIV-1 Tat-activation. *J. Mol. Biol.* **277**(2), 179–197.

Yik, J. H., Chen, R., Pezda, A. C., Samford, C. S., and Zhou, Q. (2004). A human immunodeficiency virus type 1 Tat-like arginine-rich RNA-binding domain is essential for HEXIM1 to inhibit RNA polymerase II transcription through 7SK snRNA-mediated inactivation of P-TEFb. *Mol. Cell Biol.* **24**(12), 5094–5105.

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**(5647), 1056–1060.

Yuan, J., Cahir-McFarland, E., Zhao, B., and Kieff, E. (2006). Virus and cell RNAs expressed during Epstein-Barr virus replication. *J. Virol.* **80**(5), 2548–2565.

Yung, E., Sorin, M., Pal, A., Craig, E., Morozov, A., Delattre, O., Kappes, J., Ott, D., and Kalpana, G. V. (2001). Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1. *Nat. Med.* **7**(8), 920–926.

Zamore, P. D., and Haley, B. (2005). Ribo-gnome: The big world of small RNAs. *Science* **309**(5740), 1519–1524.

Zhang, L., Kovalev, G. I., and Su, L. (2006). HIV-1 infection and pathogenesis in a novel humanized mouse model. *Blood* **109**(7), 2978–2981.

Zhang, S., Feng, Y., Narayan, O., and Zhao, L. J. (2001). Cytoplasmic retention of HIV-1 regulatory protein Vpr by protein-protein interaction with a novel human cytoplasmic protein VprBP. *Gene* **263**(1–2), 131–140.

Zhou, M., Halanski, M. A., Radonovich, M. F., Kashanchi, F., Peng, J., Price, D. H., and Brady, J. N. (2000). Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. *Mol. Cell Biol.* **20**(14), 5077–5086.

Que Dang and Vanessa M. Hirsch

Laboratory of Molecular Microbiology, NIAID, NIH 4 Center Drive Bethesda, Maryland 20892

Rapid Disease Progression to AIDS due to *Simian immunodeficiency virus* Infection of Macaques: Host and Viral Factors

I. Chapter Overview

Simian immunodeficiency virus (SIV)-infection of nonhuman primates provides a relevant animal model system to study the pathogenesis of AIDS and to evaluate experimental therapies and vaccine strategies. A subset of SIV-infected macaques progresses rapidly to disease with transient SIV-specific immune responses and high plasma viremia. This is a relatively

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56012-6

rare occurrence in HIV-infected humans. In this chapter, we summarize the virological, immunologic, and pathological characteristics of these animals. Rapid progressor (RP) macaques appear to result from an irreversible, massive, early virus-induced killing of memory $CD4^+$ T cells, resulting in a failure to maintain immune responses and the evolution of macrophage-tropic and neutralization-sensitive variants. The pathogenesis appears to be restricted to direct cytopathic effects of SIV on the immune system as compared with the additional effects of immune activation-induced cell death and regenerative failure that accompanies slower disease progression. Additionally, unique envelope variants evolve in SIV-mac and SIV-sm-infected RP macaques. However, studies with molecular clones suggest that these variants are the end result of virus replication in a severely immunocompromised host rather than the direct cause of rapid progression. We speculate that a complex interplay of host and viral factors interact to produce rapid disease progression.

II. Introduction

The SIVs are a genetically diverse group of viruses that naturally infect a wide range of African primates and are the source of the *human immunodeficiency viruses* (HIV-1 and HIV-2). Primate lentiviruses detected in many African monkey species can be classified genetically into lineages based on their host of origin (Apetrei *et al*., 2004; VandeWoude and Apetrei, 2006). SIV from chimpanzees (SIV-cpz) and sooty mangabey monkeys (SIV-sm) are of particular interest since the HIV-1 and HIV-2 AIDS epidemics in humans have been found to originate from them, respectively (Chen *et al*., 1996; Hahn *et al*., 2000; Hirsch *et al*., 1989; Santiago *et al*., 2002). But, interestingly, natural infection of Asian monkeys such as macaques has not been detected in the wild. This restriction of primate lentiviruses to African monkeys suggests that this is an ancient virus that has coevolved with its primate host. Experimental infection of Asian macaque species with SIV-sm and SIV-mac results in an immunodeficiency syndrome that is remarkably similar to AIDS in humans (Daniel *et al*., 1985). Therefore, they have become highly relevant models for the study of AIDS (Desrosiers, 1990; Letvin *et al*., 1985) and have led to the characterization of a number of SIV isolates that are used in the study of pathogenesis, the development of vaccines, and the assessment of antiviral therapies.

Although SIV infection of macaques with pathogenic strains is fatal in the long term, there is considerable variability in disease course and rate among individuals. The majority of these macaques are conventional progressors (CPs), while 10–30% are rapid progressors (RPs) who progress rapidly to disease. RP has not been generally the primary focus of study since the association of rapid disease progression with SIV encephalitis

(SIVE) has diverted attention from the unique features of this disease course. Therefore, this chapter will discuss SIV-induced AIDS with a focus on RPs and their distinct characteristics in terms of the immunologic effects, target cells, viral evolution, and pathology.

III. SIV Strain Diversity

Many of the SIV-sm and SIV-mac isolates that have been used for pathogenesis studies in primates are described in Table I. These vary in pathogenicity for macaques from the highly attenuated, SIV-mac1A11, to the highly pathogenic strains such as SIV-mac239, SIV-mac251, and SIV-smE660. The SIV-smPBj isolate (Dewhurst *et al*., 1992; Novembre *et al*., 1993) is unique among SIV-sm/mac strains in that it induces an extremely rapid disease course with survival of less than two weeks and often as short as six days (Fultz and Zack, 1994; Fultz *et al*., 1989). In general, all of the uncloned SIV-sm/mac isolates exhibit some virulence. There are fewer examples of pathogenic molecularly cloned (or biologically cloned) SIVs that induce AIDS, and the two most widely used are SIV-mac239 (Kestler *et al*., 1988) and SIV-smE543-3 (Hirsch *et al*., 1998). However, most of the molecularly cloned viruses are minimally, if at all, pathogenic. These viruses include SIV-mac1A11 (Luciw *et al*., 1992; Marthas *et al*., 1993), SIV-smH-4 (Hirsch *et al*., 1989), SIV-macBK-28 (Edmondson *et al*., 1998), SIV-sm62d (Hirsch *et al*., 1998), SIV-mac17E-Fr (Anderson *et al*., 1993; Zink *et al*., 1997), and SIV-mneCl8 (Kimata *et al*., 1999; Rudensey *et al*., 1995).

IV. Biology of SIV

SIV and HIV-1 share many properties, including genetic structure, gene regulation, cell tropism, and cellular receptor usage. The tropism and cell receptors used by these viruses are critical in determining their association with immunodeficiency. Both of these viruses use CD4 as a receptor and therefore infect $CD4^+$ T lymphocytes and macrophages. In addition, both viruses use the chemokine receptor molecule, CCR5 (R5), for viral entry. R5 variants dominate early in HIV infection and CXCR4 (X4) variants can emerge later in disease (Berger *et al*., 1999; Zhang *et al*., 1998). It is still controversial whether the emergence of X4 variants is the result or cause of the eventual disease progression in these patients. However, it is clear that the emergence of X4 variants is not required for disease progression since only R5 variants are detected in many AIDS patients (Berger *et al*., 1999). While changes in the V3 region of envelope of HIV-1 is associated with the emergence of variants with CXCR4 coreceptor preference (Koot *et al*., 1993; Schuitemaker *et al*., 1992), changes in the analogous cysteine loop

TABLE I Genetic and Pathogenic Diversity of SIV Strains

Subtype	*Source*	*Strain*	*Isolate/form*	*Disease potential*	*Other comments*
SIV-mac	NENPRC	SIV-mac251	Uncloned	High, AIDS	RP with CD8 depletion
		SIV-mac182	Uncloned	High, NeuroAIDS	RP with CD8 depletion
		SIV-mac32H	Uncloned	Moderate, AIDS	
		SIV-macJ5	Molecular clone	Low, AIDS	
		SIV-macBK28	Molecular clone	Low, AIDS	
		SIV-mac1A11	Molecular clone	Attenuated	
		SIV-mac239*	Molecular clone	High, AIDS	
		SIV-mac316*	Molecular clone	Moderate, AIDS	
		SIV-mac17E-Fr*	Molecular clone	High, NeuroAIDS	RP in combination with B670
		SIV-mac155T3*	Molecular clone	Moderate, AIDS	CXCR4 tropic
	WaNPRC	SIV-mne	Uncloned	Moderate, AIDS	
		SIV-mneE11S	Biological clone	Low, AIDS	
		SIV-mne-cl8	Molecular clone	Low, AIDS	
		SIV-mne170	Molecular clone	Moderate, AIDS	
SIV-sm	TNPRC	SIV-smDeltaB670	Uncloned	High, AIDS	RP in combination with 17E-Fr
		SIV-smF236	Uncloned	Moderate, AIDS	
		SIV-smH-4	Molecular clone	Low, AIDS	
		SIV-smH-3	Molecular clone	Low, AIDS	
		SIV-sm62d*	Molecular clone	Low, AIDS	
		SIV-smE660*	Uncloned	High, AIDS	
		SIV-smE543	Uncloned	High, AIDS	
		SIV-smE543-3	Molecular clone	High, AIDS	
		SIV-smH635-FC*	Molecular clone	Moderate, AIDS	
	YNPRC	SIV-smm9	Uncloned	Moderate, AIDS	
		SIV-smPBj14	Biological clone	High, AIDS	Acutely lethal
		SIV-smPBj6.6	Molecular clone	High, AIDS	Acutely lethal
		SIV-smFGb	Uncloned	High, NeuroAIDS	RP in PT macaques
		SIV-smPGm/mln*	Uncloned	High, NeuroAIDS	RP in PT macaques

*Derived from a rapid progressor.
NENPRC = New England National Primate Research Center; WaNPRC = Washington NPRC; TNPRC = Tulane NPRC; YNPRC = Yerkes NPRC; RP = rapid progressors.

in SIV are rare (Hirsch *et al*., 1994b; Johnson and Hirsch, 1992; Kodama *et al*., 1993; Overbaugh *et al*., 1991). The vast majority of SIV isolates do not use the X4 receptor; and therefore, the disease process in SIV infection of macaques is primarily an R5-driven disease process (Chen *et al*., 1997; Edinger *et al*., 1997a; Kunstman *et al*., 2003).

The use of CD4 and CCR5 as cellular receptors has proven to be critical in understanding the pathogenesis of HIV- and SIV-induced disease since it explains much of the unique effects of these viruses (Brenchley *et al*., 2004; Mattapallil *et al*., 2005; Nishimura *et al*., 2004; Picker, 2006; Picker *et al*., 2004). CCR5 is expressed on memory T cells that have previously encountered antigen. This includes short-lived effector cells and central memory cells that can be activated to proliferate on encountering antigen. Both of these cell types are abundant in effector sites such as mucosal tissues, including the gastrointestinal tract and lung. In contrast, X4 is expressed on naive T cells, antigen-naive cells emerging from the thymus that are abundant in blood and secondary lymphoid tissues such as lymph nodes. Therefore, the major effect of SIV and HIV during acute infection appears to be on memory $CD4^+$ T cells at mucosal sites (Brenchley *et al*., 2004; Veazey *et al*., 1998, 2000).

V. Pathogenesis of SIV in Macaques

A. SIV-sm and SIV-mac Infection as Models for AIDS

Our understanding of the pathogenesis of HIV- and SIV-induced AIDS is still an evolving process. It has become apparent that the disease process cannot be explained by a simple loss of $CD4^+$ T cells through direct virus killing. With the exception of the acute phase of infection, the proportion of cells infected at any one time point is too low to account for the CD4 loss and the disease course is too protracted. There is also accumulating evidence that immune activation-induced cell death and exhaustion of the regenerative and homeostatic processes contribute significantly to the eventual development of clinical disease (Douek, 2003; Douek *et al.,* 2003; Finkel and Banda, 1994; Hazenberg *et al*., 2000; Lackner and Veazey, 2007; Moss and Bacchetti, 1989; Picker, 2006). The use of animal models such as SIV infection of macaques is a key element in delineating the disease process.

The pathogenesis of SIV-sm/mac infection in macaques appears to be remarkably similar to human AIDS. Both show a period of intense viremia during the first four weeks of infection (primary) with rapid dissemination of virus to lymphoid and mucosal sites. The primary phase of infection is associated with a sharp decline in memory $CD4^+$ T cells, particularly in mucosal sites such as the gastrointestinal tract (Lackner and Veazey, 2007; Mattapallil *et al*., 2005; Veazey *et al*., 1998) and the numbers of $CD4^+$ T cells in

mucosal sites never fully recover. The development of a cytotoxic T lymphocyte (CTL) (Koup *et al.*, 1994; Schmitz *et al.*, 1999) and neutralizing antibody response is associated with modulation in viremia to varying degrees depending on the individual. Primary infection is followed by an asymptomatic period, characterized by persistent viral replication, lymphadenopathy, immune activation, strong humoral and cellular immune responses, and a slow but progressive decline in numbers of circulating $CD4^+$ T lymphocytes. Neutralizing antibody and CTL escape mutants (Barouch *et al.*, 2003; Friedrich *et al.*, 2004; Koup, 1994) develop throughout infection and presumably contribute to the eventual progression to disease. Opportunistic infections (OIs) and clinical AIDS are generally observed when peripheral CD4 counts drop to less than 200 cells/μl. Animals die from a variety of OIs such as rhesus *Cytomegalovirus* (CMV), *Pneumocystis* pneumonia, and *Mycobacterium avium* infection (Baskin *et al.*, 1988; McClure *et al.*, 1989; Simon *et al.*, 1994; Zhang *et al.*, 1988). Similar to HIV, SIV can also result in neuro-AIDS due to SIVE (Baskin *et al.*, 1992; O'Neil *et al.*, 2004; Orandle *et al.*, 2001; Ringler *et al.*, 1988; Westmoreland *et al.,* 1998; Zink *et al.*, 1997).

B. Variation in Disease Progression

SIV infection of macaques causes a significantly more rapid disease course than HIV-1. The disease course in HIV-infected patients is highly variable ranging from long-term asymptomatic survival for over 15 years (Cao *et al.*, 1996; Dean *et al.*, 1996; Migueles and Connors, 2002; Pantaleo *et al.*, 1995) to rapid progression to AIDS within 1 or 2 years of infection (Garland *et al.*, 1996; Michael *et al.*, 1997; Montagnier *et al.*, 1997). In contrast, median survival in SIV-infected macaques ranges from 1 to 2 years (Hirsch and Johnson, 1994; Lackner and Veazey, 2007; Letvin and King, 1990; Simon *et al.*, 1994), compared to the median 10-year survival for untreated HIV infection in humans. Even within macaques inoculated with a common SIV strain, the disease course varies considerably from rare, long-term nonprogression to rapid progression (Evans *et al.*, 1999; Hirsch *et al.*, 1996; Lifson *et al.*, 1997; Staprans *et al.*, 1999). The majority of SIV-sm/mac-infected macaques progress to AIDS in one to three years with moderate-to-high viremia. We have designated these as CPs due to their similarity to AIDS in humans (Brown *et al.*, 2007). At the most extreme end of the spectrum are a subset of animals that progress to disease in less than six months from the time of inoculation (Fig. 1). This group comprises 10–30% of animals inoculated with pathogenic strains (Baskin *et al.*, 1988; Hirsch *et al.*, 2004; Orandle *et al.*, 2001; Picker *et al.*, 2004; Ryzhova *et al.*, 2002). It is fairly rare to observe long-term survivors (long-term nonprogressors) of infection with highly pathogenic strains, and these will not be discussed in this chapter.

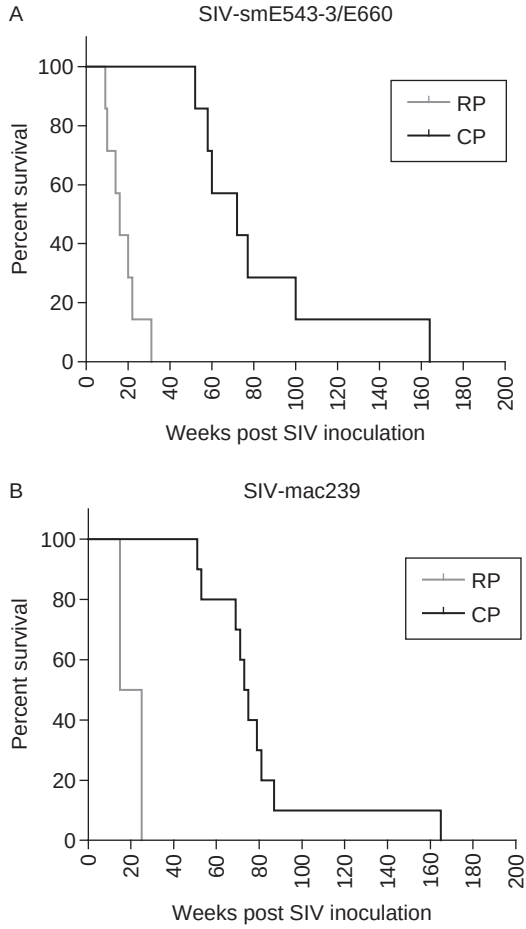


FIGURE 1 Similar survival rates for SIV-infected RP macaques in Kaplan–Meier survival plot. (A) Macaques infected with SIV-smE543-3/E660. (B) Macaques infected with molecularly cloned SIV-mac239. RP macaques are indicated as gray lines and CP macaques are indicated as black lines.

C. Acutely Lethal SIV-smPBj

In contrast to the majority of SIV isolates, the SIV-smmPBj virus isolated from a pigtailed macaque (PBj) infected with the AIDS-inducing SIV-smm9 strain had evolved the novel ability to replicate in and induce proliferation of unstimulated macaque PBMC *in vitro* (Fultz *et al*., 1989). Additionally, an acute and lethal illness was observed within 14 days of *in vivo* inoculation, characterized by diarrhea, dehydration, and severe lymphopenia (Dewhurst *et al*., 1992; Fultz and Zack, 1994; Fultz *et al*., 1989). Pathological features included major gastrointestinal cytopathology, massive mononuclear cell

infiltration within the gastrointestinal tract, high levels of virus replication, and immune system hyperactivation (Fultz and Zack, 1994). Elevated levels of cytokines such as tumor necrosis factor alpha and interleukin-6 produced within the sites of the lesions (Du *et al.*, 1995) suggested that the pathogenesis of this novel disease syndrome was cytokine mediated. While multiple genes may contribute to the overall virulence of SIV-smPBj, the principle pathogenic determinant appears to be due to an R17Y mutation within Nef. This substitution introduces an immunoreceptor tyrosine-based activation motif (ITAM) into the amino terminus of the SIV-smPBj Nef protein. Introduction of this motif into Nef of the AIDS-inducing SIV-mac239 molecular clone confers the ability of the resulting virus to replicate in unstimulated PBMC and to induce acute disease in macaques (Du *et al.*, 1995; Novembre *et al.*, 1994). Conversely, substitution of Y17 in acutely lethal SIV-smPBj clones abrogates the acute pathogenesis (Du *et al.*, 1995). While this is an interesting syndrome, there does not appear to be parallel variants and syndromes in HIV-infected people. Therefore, this virus system is not used extensively in pathogenesis or vaccine studies and will not be discussed further in this chapter.

D. Clinical Correlates of Disease Progression

Over the last 10 years, a number of correlates have been identified in HIV-infected humans and SIV-infected macaques that are predictive of the rate of disease progression. These include: (1) $CD4^+$ T cells in blood and mucosal sites, (2) plasma viral load, and (3) the degree of immune activation.

1. $CD4^+$ T Cells in Blood and Mucosal Sites

The initial predictors of absolute $CD4^+$ T cells in the circulation or the ratio of $CD4^+$ to $CD8^+$ T cells do not have very good predictive value since these tend to be a late event in disease progression. However, recent studies of SIV-infected macaques revealed that the extent of depletion of memory $CD4^+$ T-cell subsets is a valuable predictor of disease progression. Veazey *et al.* (1998) initially demonstrated selective depletion of mucosal $CD4^+$ T cells in the gastrointestinal tract. It has since become apparent that such CD4 depletion similarly affects other mucosal sites such as the lung and reproductive tract (Mattapallil *et al.*, 2005; Picker *et al.*, 2004). Studies of coreceptor expression and subset analysis of $CD4^+$ T cells subsequently revealed that mucosal $CD4^+$ T cells are activated memory cells that selectively express CCR5 (Nishimura *et al.*, 2004; Pitcher *et al.*, 2002). These cells are massively infected in the initial stages of infection and apparently eliminated by the virus (Mattapallil *et al.*, 2005). The degree of memory $CD4^+$ T-cell depletion is predictive of the rate of disease progression with marked loss being associated with rapid disease (Nishimura *et al.*, 2007). Interestingly, prior vaccination results in a significant preservation of central memory $CD4^+$ T cells and associated improved survival (Letvin *et al.*, 2006; Mattapallil *et al.*, 2006).

2. *Plasma Viral Load*

Plasma viral load has also become the mainstay predictor of disease progression and a useful indicator of when to initiate antiretroviral therapy. Similar to HIV infection in humans (Mellors *et al.*, 1996, 1997; Piatak *et al.*, 1993), the levels at which viremia plateaus following primary viremia is predictive of disease outcome in SIV infection (Hirsch *et al.*, 1996; Watson *et al.*, 1997). Thus, macaques with low levels of viremia remain disease-free longer than macaques with high levels of viremia (Hirsch *et al.*, 1996; Lifson *et al.*, 1997; Staprans *et al.*, 1999; Watson *et al.*, 1997), and prior vaccination may result in a reduction in plasma viremia (Ourmanov *et al.*, 2000).

3. *Immune Activation*

Finally, both HIV and SIV infections in humans and macaques, respectively, are associated with intense immune activation as indicated by persistent lymphadenopathy, high rates of T-cell proliferation, high T-cell turnover, and increased expression of activation markers such as CD38 and CD69. *In vivo* labeling of T cells in HIV-infected patients demonstrated increased numbers of rapidly cycling cells (Kovacs *et al.*, 2001; Mohri *et al.*, 2001). The rate of turnover is proportional to the level of viremia and markers of activation, and T-cell turnover is predictive of disease progression in untreated HIV-infected patients. Antiretroviral therapy decreases both the level of activation and viremia (Anthony *et al.*, 2003) consistent with the hypothesis that generalized T-cell activation contributes to disease progression (Deeks and Walker, 2004; Douek *et al.*, 2003; Hazenberg *et al.*, 2000). Indeed, some studies have shown that the extent of immune activation is actually a better predictor of the rate of disease progression than viral load (Deeks *et al.*, 2004; Giorgi *et al.*, 1999; Rodriguez *et al.*, 2006). The degree of immune activation is one of the major differences between pathogenic SIV-sm/mac infection of macaques and natural infection of sooty mangabeys. Asymptomatic SIV-sm-infected sooty mangabeys do not exhibit heightened immune activation (Chakrabarti, 2004; Silvestri, 2005; Silvestri *et al.*, 2003) consistent with the role of immune activation in disease progression.

VI. Unique Immunologic, Virological, and Pathological Features of Rapid Disease

It is tempting to consider the spectrum of disease course in SIV-infected macaques as part of a continuum sharing similar pathogenic mechanisms. However, RP macaques exhibit a number of unique clinical, immunologic, virological, and pathological characteristics that indicate different mechanisms from those occurring in conventional disease progression.

A. Immunologic Features

1. Transient Immune Responses

SIV-infected RP macaques are distinguished from CPs by transient antibody responses in the presence of persistent high viremia. This phenomenon was first reported in macaques inoculated with SIV-sm strains at the Tulane Primate center. Such animals characteristically showed persistence of plasma viral antigen (Zhang *et al*., 1988). RP macaques mount an initial humoral and cellular immune response at the appropriate time following infection, but these responses wane rapidly within the first three to four weeks of infection (Hirsch *et al*., 2004). The lack of SIV-specific antibody responses is a common theme among RP macaques, regardless of the strain used for inoculation (O'Neil *et al*., 2004; Zhang *et al*., 1988). The subsequent immune defect in these animals affects both cellular and humoral immunity specific to SIV, as well as immune responses against unrelated antigens (Hirsch *et al*., 2004).

2. Irreversible Depletion of Memory $CD4^+$ T Cells

Surprisingly considering the rapidity of the disease course in these animals, the total numbers of $CD4^+$ T cells in the peripheral blood are relatively unaffected (Hirsch *et al*., 2004). However, recent studies have shown that RP macaques suffer an early massive loss of memory $CD4^+$ T cells in the blood, lymphoid tissues, and particularly in mucosal sites such as the gastrointestinal tract (Nishimura *et al*., 2004, 2007; Picker *et al*., 2004). While significant loss of memory $CD4^+$ T cells occurs in all macaques during the primary phase of SIV infection (Li *et al*., 2005; Mattapallil *et al*., 2005; Nishimura *et al*., 2004; Picker, 2006; Picker *et al*., 2004; Veazey *et al*., 1998), the loss is profound and irreversible in RP macaques. Studies by Picker *et al.* suggest that the lack of tissue delivery of new memory $CD4^+$ T cells is the mechanism underlying immune failure in RP macaques. Naive $CD4^+$ T cells are relatively preserved terminally in RP macaques; this contrasts with a generalized loss of both memory and naive $CD4^+$ T cells in CP macaques as well as HIV-infected humans. The generalized loss in CP macaques likely reflects the long-term effects of virus and immune activation-induced cell death on the regenerative process of $CD4^+$ T cells (Nishimura *et al*., 2007) and suggests that different mechanisms may be at work in CP macaques as compared to RP macaques.

3. Effects on Immune Activation

In general, RP macaques show a lack of proliferation and immune activation normally observed in SIV infection of macaques. This is evident by the progressive loss of Ki-67 expressing $CD4^+$ and $CD8^+$ T cells (Nishimura *et al*., 2007; Picker *et al*., 2004). Lymph node pathology is distinctive with no evidence of the lymphadenopathy generally associated

with SIV infection (Chalifoux *et al.*, 1987; Simon *et al.*, 1994). Instead, depletion of B cells from germinal centers of lymph nodes and disruption of the follicular dendritic cell network have been observed (Zhang *et al.*, 2007). Both the loss of memory $CD4^+$ T cells and the disruption to germinal centers are likely to contribute to the failure of these animals to maintain SIV-specific immune responses.

B. Virological Features

Plasma viremia, as measured by viral load, is always at the high end of the spectrum in RP macaques, as compared to CPs, but there is an overlap in the ranges during primary infection (Brown *et al.*, 2007). However, late-stage viremia is significantly higher in RP macaques. As shown in Fig. 2, higher viremia is a feature of both SIV-mac239 and SIV-smE543-3/E660-induced rapid disease progression. There is also evidence that the rate of increase in plasma viremia during primary infection is a predictor of disease course (Lifson *et al.*, 1997; Staprans *et al.*, 1999). However, most studies in SIV-infected macaques do not take the daily samples necessary to see such effects and since the peak of viremia is transient, it is difficult to get an accurate assessment of peak viremia in most SIV-infected macaques. As we will discuss later, the virus in RP macaques also appears to undergo unique evolution.

C. Pathological Features

Since the initial reports of SIV infection of macaques, there have been a number of comprehensive reports and reviews on the pathology of SIV infection (Baskin *et al.*, 1988, 1992; Letvin and King, 1990; McClure *et al.*, 1989; Simon *et al.*, 1992; Wyand *et al.*, 1989). These studies have included animals spanning the entire range of the disease spectrum, without focusing on lesions that might be specific to RPs. All reports included a number of animals with lesions that appeared to be a primary effect of SIV-replication, characterized by the presence of numerous multinucleated giant cells (MNGCs) in lymphoid tissues (Hirsch *et al.*, 1991; Ringler *et al.*, 1988).

Recently, we conducted a retrospective study to compare the pathology in RP and CP macaques (Brown *et al.*, 2007). The end-stage tissues of RP macaques were observed to exhibit primary SIV-induced pathological lesions in lymphoid tissues as well many other nonlymphoid tissues. As shown in Fig. 3, MNGCs were frequently observed in the lung and brain as well as other organs of RP macaques. These cells expressed SIV as shown by *in situ* hybridization and were identified as macrophages by confocal microscopy (Fig. 4). Despite evidence of immune suppression, OIs were rarely observed in RP macaques, and thus their severe disease was presumably due to a direct effect of SIV-replication in tissues.

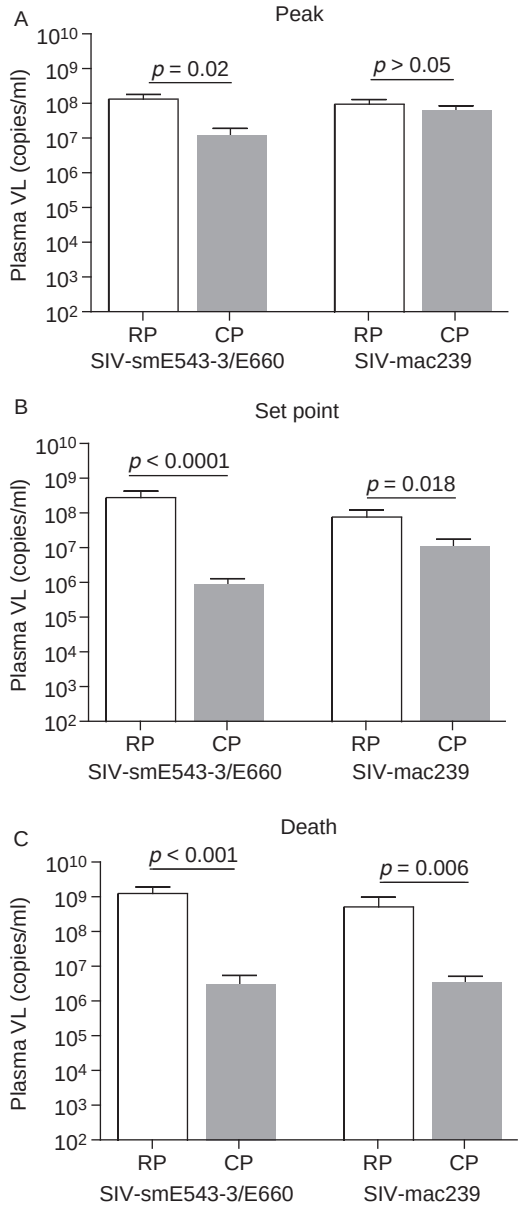


FIGURE 2 Plasma viremia of macaques infected with SIV-smE543-3/E660 or SIV-mac239 at: (A) Peak plasma viral load (VL), (B) Plasma viral load at set point, and (C) Plasma viral load at death. RP macaques are shown as white bars and CP macaques are shown as gray bars. The error bars represent the standard error of the mean. Mann–Whitney test was performed to determine statistical significance.

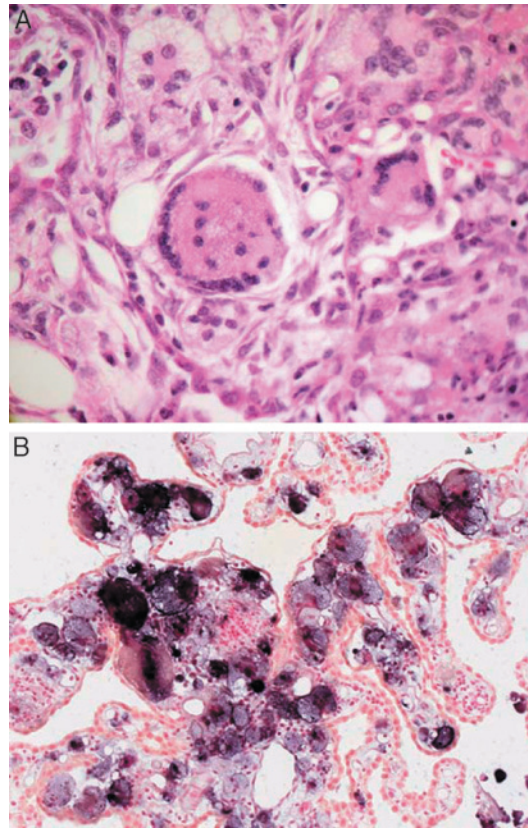


FIGURE 3 Histopathology and SIV-specific *in situ* hybridization of multinucleated giant cells in the lung (A) and choroid plexus of the brain (B) of a RP showing that these cells express SIV viral RNA.

In contrast, the clinical disease in CP macaques could often be attributed to infections with opportunistic pathogens, a pathological picture reminiscent of HIV-induced AIDS in humans. Virus expression was restricted to lymphoid tissues in CP macaques, and the primary target cells by end-stage disease were T cells (Brown *et al.*, 2007).

The lack of OIs in RP in our study is not uniform among RP macaques inoculated with other strains of SIV. CMV reactivation appears to be common in RP macaques inoculated with SIV-mac239 (Kaur *et al.*, 1996, 2002, 2003). In addition, opportunistic agents have been identified in pigtailed macaques inoculated with the neurovirulent SIV-smPGm/mln isolate (O'Neil *et al.*, 2004) and rhesus macaques inoculated with SIV-mac239 or 251 (Ringler *et al.*, 1988; Westmoreland *et al.*, 1998). Thus, there may be differences in the presentation of this syndrome in different SIV-macaque models.

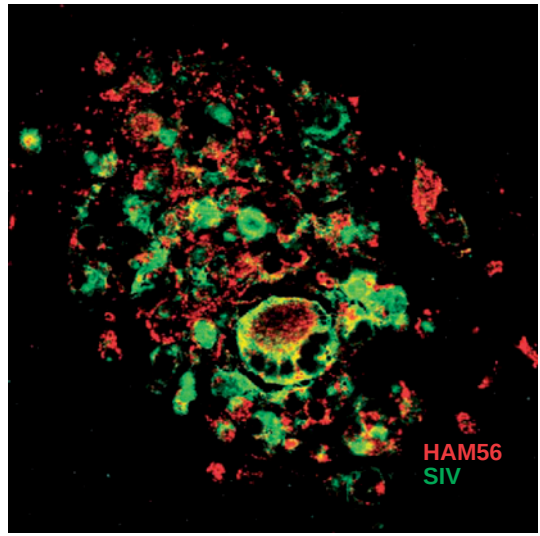


FIGURE 4 Confocal microscopy to identify the target cells of SIV in lesions in the brain of RP macaques. Red indicates HAM56, a marker for macrophages and green indicates SIV RNA. SIV-expressing cells coexpress HAM56, indicating that they are macrophages.

Another characteristic feature of RP macaques was an increased preference for terminal infection of macrophages over T cells. This was manifested by the presence of MNGCs and the predominance of SIV-expressing macrophages in the lung and brain of these animals (Brown *et al.*, 2007). The apparent switch from infection of memory $CD4^+$ T cells early in infection to macrophages late in infection can be explained by the initial severe depletion of T cells targeted by SIV.

Studies of SIV neuropathogenesis have the greatest focus on RPs since SIVE is strongly associated with high viremia, poor humoral immune responses, and rapid disease progression (O'Neil *et al.*, 2004; Westmoreland *et al.*, 1998). RP macaques in our retrospective study uniformly exhibited SIVE (Brown *et al.*, 2007), whereas SIV-induced lesions were rare in the brains of CPs. This observation differs from what is seen in SIV-mac-inoculated RP rhesus macaques. While SIVE is strongly associated with rapid progression, there are exceptions. Thus, not all SIV-mac-infected RP macaques exhibit SIVE and moreover, some SIV-mac-infected CP macaques will (Ringler *et al.*, 1988; Westmoreland *et al.*, 1998). SIVE is more frequently observed in SIV-mac251 than in the T-tropic SIV-mac239 (Ringler *et al.*, 1988). In contrast, almost all pigtailed macaques inoculated with the combination of the neurovirulent SIV-mac17E-Fr and uncloned SIV-smB670 are RPs with SIVE (Zink *et al.*, 1997, p. 384). Similarly, over 90% of PT macaques inoculated with the neurovirulent SIV-smPGm/mln (O'Neil *et al.*, 2004) progress rapidly and

develop SIVE. Therefore, there are obviously a number of host and viral factors that may influence the progression of disease.

VII. Host Factors That Influence Disease Progression

A. Macaque Species

In studying SIV infection in monkeys, the three most widely available macaque animal models are the pigtail (*Macaca nemestrina*), cynomolgus (*Macaca fascicularis*), and rhesus (*Macaca mulatta*). However, these three species of macaques vary in their rate of disease progression, following experimental infection with SIV. Pigtail macaques have been observed to be the most susceptible and progress to disease more rapidly than the cynomolgus which in turn is more susceptible than the rhesus macaques (Frumkin *et al*., 1993; Hirsch *et al*., 1995; Lewis *et al*., 1992; Rosenberg *et al*., 1991). Within the rhesus species, the most commonly used in experimental studies are rhesus macaques of Indian origin; however, macaques of Chinese origin have also been used. Interestingly, despite belonging in the same species, Chinese rhesus macaques were found to differ in their ability to control SIV infection with a lower plasma viral load set point, significantly longer survival rates, and fewer clinical disease symptoms when compared to the Indian rhesus macaques (Joag *et al*., 1994; Ling *et al*., 2002; Trichel *et al.,* 2002). Thus, the species as well as subspecies should be taken into account when designing and interpreting experiments using nonhuman primates as animal models.

B. Host Immune Responses and Major Histocompatibility Complex

Host immune responses such as CTL and their interaction with the major histocompatibility complex (MHC) class I molecules have been shown to be important in the rate of disease progression. The MHC class I molecules present intracellularly processed antigenic epitopes to T cells resulting in initiation of the removal of foreign antigens (Parham and Ohta, 1996). The genes that encode these molecules have been found to be highly polymorphic, generating a highly diverse group of molecules (Mattapallil *et al*., 2005; Otting *et al*., 2005; Parham and Ohta, 1996). This results in a greater breadth of antigenic peptides recognized by CTL that, in turn, can control viremia and slow down disease progression. In monkeys, the most well characterized and studied is the Mamu-A-*01 molecule (Miller *et al*., 1991) found in ~20–30% of Indian rhesus macaques. Studies have shown that in SIV-infected rhesus macaques expressing these molecules as compared to animals that were Mamu-A-*01 negative, there was a significantly

lower set point of viral load, indicating control of viral replication and slow disease progression, resulting in a prolonged survival time (Muhl *et al.*, 2002; O'Connor *et al.*, 2003). The MHC class I molecule has also been shown to be important in the disease outcome of HIV-infected humans where HLA-B*57 and HLA-B*27 expressions in Caucasians can delay the onset of AIDS (Kaslow *et al.*, 1996). Conversely, expression of HLA-B35 has been found to be associated with accelerated progression to AIDS (Itescu *et al.*, 1992). The polymorphism exhibited by this class of genes implies that heterozygosity at these loci will increase the repertoire of antigens recognized by CTL. Therefore, if an individual is HLA homozygous, there is a limit on the variety of class I molecules and on the recognition of antigenic peptides. Indeed, the concept of homozygosity at the HLA class molecules has been demonstrated to be associated with the acceleration of disease (Carrington *et al.*, 1999; Tang *et al.*, 1999). This has also been observed in rhesus macaques where homozygosity for MHC class II molecule Mamu-DQB1*0601 can predispose rhesus macaques for rapid disease progression following infection with SIV (Sauermann *et al.*, 1997, 2000). Interestingly, homozygosity for a deletion of 32-base pairs in the CCR5 gene results in a truncated, dysfunctional protein that does not get expressed on the cell surface, and thus, individuals who are homozygous at this allele are protected against HIV infection (Dean *et al.*, 1996; Liu *et al.*, 1996).

Clearly, immunogenetics can influence the rate of disease progression, but the role of CTL (through the MHC-I-restricted process) is also important. Experiments have shown that transient depletion of $CD8^+$ T cells in rhesus macaques causes a rapid and high increase in viral replication that can result in acceleration of disease progression. The high viremia is subsequently suppressed following the reemergence of the $CD8^+$ T cells, demonstrating that CTL plays an important role in pathogenesis (Jin *et al.*, 1999; Schmitz *et al.*, 1999). In fact, when $CD8^+$ T cells are depleted and macaques are inoculated with SIV-mac182, rapid disease progression and SIVE are consistently induced (Roberts *et al.*, 2003).

VIII. Role of Virus Genotype/ Phenotype in Rapid Disease Progression

A. Specific Genes Involved in Pathogenesis

Many of the accessory genes of SIV such as *Vpr*, *Vpx*, *Nef*, and *Vif* are involved in pathogenesis of SIV in macaques and deletion of these results in attenuation (Desrosiers *et al.*, 1998; Hirsch *et al.*, 1998; Kestler *et al.*, 1991). In particular, Nef, among many other functions, has been shown to down-regulate expression of MHC class I molecules (recently reviewed in Roeth and Collins, 2006). This protein, while dispensable for viral replication

in vitro, is necessary for pathogenesis *in vivo* (Kestler *et al.*, 1991). As mentioned earlier, the R17Y mutation in Nef has been shown to induce extreme rapid disease progression (Du *et al.*, 1995). However, the deletion of Nef results in low pathogenicity, with animals infected with the SIV-mac239Δnef variant remaining healthy for years (Alexander *et al.*, 2003). Furthermore, Δnef variants are being tested as a possible candidate for live attenuated virus vaccine studies (Daniel *et al.*, 1992). There does not appear to be any other specific association of rapid disease progression with substitutions in the other accessory genes.

B. Evolution of SIV in RPs

1. Molecular Changes

The envelope of SIV undergoes unique molecular evolution in RP macaques. We observed a convergent pattern of substitutions in *env* in RP macaques inoculated with the cloned SIV-smE543-3 (Dehghani *et al.,* 2003; Kuwata *et al.*, 2006). Similar mutations have been observed in tissues of RP inoculated with SIV-mac239 (Anderson *et al.*, 1993; Kodama *et al.*, 1993; Mori *et al.*, 1992); and therefore, this appears to be an evolutionary pattern specifically associated with rapid disease. This constellation of unique substitutions include: (1) loss of a highly conserved potential glycosylation site in the V1/V2 region (N158D/S or S160N/G), (2) substitutions in the V3 analogue (P337T/S/H/L and R348W), and (3) substitutions in the highly conserved GDPE motif (G386R and D388N/V). Two of the motifs that are altered in RP viruses are highly conserved in SIV and/or HIV and the third (V3) is highly conserved in SIV. Some of the specific changes observed in SIV-mac239 and SIV-smE543-3-inoculated RPs are shown in Fig. 5.

2. Biological Properties of Virus from RP Macaques

Common features between clones with RP-associated mutations are CD4-independent use of CCR5, macrophage tropism, and increased sensitivity to neutralizing antibodies (Means *et al.*, 2001; Mori *et al.*, 2000; Puffer *et al.*, 2002). The envelope substitutions described above were associated with the acquisition of CD4-independent usage of CCR5 in cell-fusion assays (Bhattacharya *et al.*, 2003; Dehghani *et al.*, 2003; Edinger *et al.*, 1997b; Mori *et al.*, 2000; Ryzhova *et al.*, 2002). In particular, the substitutions in the V3 loop analogue of SIV are associated with changes in tropism *in vitro* and with CD4-independent usage of CCR5 (Edinger *et al.*, 1997b; Hirsch *et al.*, 1994a). Previous studies of SIV and HIV have also shown that the GDPE motif is critical for interaction with the CD4 molecule, apparently by direct binding to the CD4 receptor molecule; therefore, mutations in this region significantly reduce the ability of virus to bind CD4 and thus impair

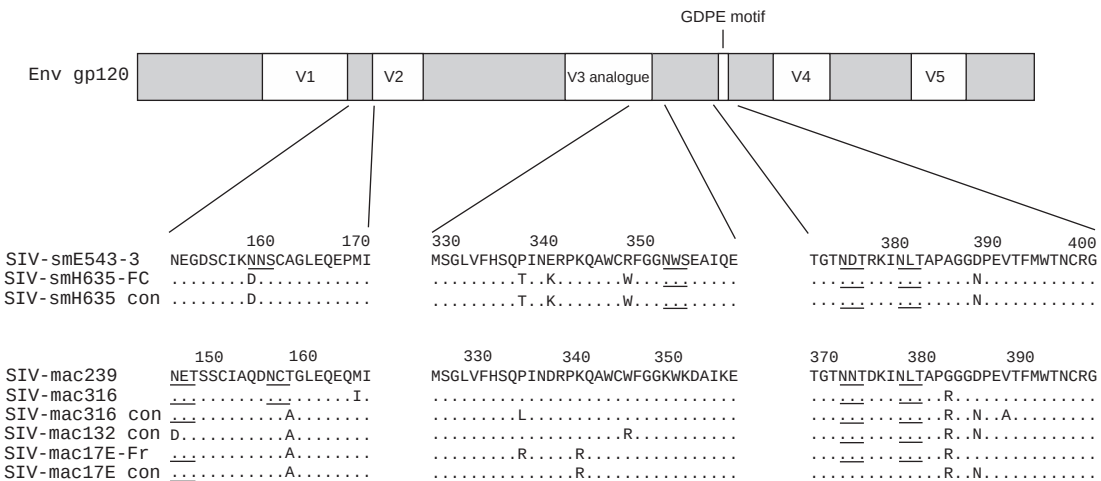


FIGURE 5 Rapid disease-associated mutations in SIV envelope gp120 region. Selected sequences show common substitutions in gp120 region in RP macaques. Parental sequences of either SIV-smE543–3 or SIV-mac239 are shown at the top for comparison. Dots indicate identity and potential N-linked glycosylation sites are underlined. Con = consensus sequence.

infectivity. Such CD4-independent SIVs include SIV-mac316 (Mori *et al.*, 2000) and SIV-mac17E-Fr (Anderson *et al.*, 1993; Edinger *et al.*, 1997b).

Although this combination of unusual substitutions is dominant in tissues and plasma of RP macaques infected with either SIV-sm (Campbell and Hirsch, 1994; Dehghani *et al.*, 2003; Hirsch *et al.*, 1994b) or SIV-mac (Anderson *et al.*, 1993; Kodama *et al.*, 1993; Mori *et al.*, 1992), it has proven difficult to derive infectious clones that contain a combination of the mutations. Thus, the SIV-mac RP clones, SIV-mac316, and SIV-mac17E-Cl lack the full complement of RP-specific mutations observed in the animals from which they were cloned (see Fig. 5). Full-length clones from an RP pigtail macaque, PT62, contained a variety of combinations of RP-specific substitutions; however, the majority of these clones replicated poorly *in vitro* particularly in primary macaque cells (Hirsch *et al.*, 1994b). Recently, we derived clones from the terminal plasma of an RP rhesus macaque, H635, which had been inoculated with an SIV-smE543–3 derivative. These clones differed from SIV-smE543-3 at seven positions and replicated inefficiently in primary macaque PBMC and monocyte-derived macrophages *in vitro* (Kuwata *et al.*, 2006). Our data (Kuwata *et al.*, 2006) and these previous studies with SIV-mac239 RP clones suggest that the specific mutations in envelope result in a virus that is less fit to replicate *in vitro* in primary macaque cells.

C. *In Vivo* Studies of the Role of Virus in Rapid Disease

Are host factors responsible for driving disease progression or is the viral variant responsible? If more pathogenic strains evolve during infection, one would then expect that the passage of these variants would accelerate disease progression in the recipient hosts. There is evidence from a number of studies that more virulent variants evolve in SIV-infected macaques. For example, studies of the moderately pathogenic SIV-mne clone have demonstrated the evolution of more pathogenic variants during disease progression (Kimata *et al.*, 1999; Rudensey *et al.*, 1995). In addition, sequential macaque passage of pathogenic SIV-smDeltaB670 resulted in shortened survival and reproduced a rapid disease phenotype (Holterman *et al.*, 1999). At least one SIV-sm isolate, SIV-smFGb and its derivative SIV-smPGm/mln, can reproducibly recapitulate rapid disease progression in pigtailed macaques (Novembre *et al.*, 1998; O'Neil *et al.*, 2004). The majority of infectious clones derived from RP macaques (SIV-mac316, SIV-mac17E-Fr, and SIV-sm62d) are actually somewhat attenuated *in vivo* (Hirsch *et al.*, 1994b; Puffer *et al.*, 2002). However, SIV-mac17E-Fr in combination with uncloned SIV-smDeltaB670 will reproduce rapid disease in conjunction with neuropathogenesis, again in pigtailed macaques (Zink *et al.*, 1997, 1999). Thus, these viruses may require a "helper" virus.

Since we observed specific variants evolving in multiple RP macaques infected with molecularly cloned virus, we evaluated this hypothesis by creating a molecular clone from an RP at terminal disease (Kuwata *et al.*, 2006). This clone, SIV-sm635FC, contained a consensus of the RP-associated mutations described above (Kuwata *et al.*, 2006). We speculated that if the mutations had evolved during the course of infection and were responsible for rapid disease progression, naive macaques inoculated with this clone would also progress rapidly. However, we did not observe this (Takeo Kuwata and V.H., unpublished data). There was a rapid reversion of the mutations, suggesting that in the face of immune pressure these mutations were selected against. Indeed, similar to other RP clones such as SIVmac316, SIV-smH635FC was significantly more sensitive to neutralizing antibody than its parent, SIV-smE543-3. Clearly, the end-stage variants were not responsible for inducing acute immune failure, the first step to rapid disease progression. Rather, we speculate that a unique interplay of the virus and host allows robust virus replication, resulting in the early destruction of memory $CD4^+$ T cells and immune failure. The subsequent disease is accompanied by expansion of RP variants that may play a role in the development of encephalitis, gastrointestinal disorders, and pneumonia through their effects on tissue macrophages.

IX. Summary

The cause of rapid progression is therefore more complex than the genotype/phenotype of the virus inoculum. Clearly, the virulence of the virus inoculum is important; attenuated or minimally pathogenic SIVs are never associated with this phenomenon. However, the host response is also equally important with innate, humoral, and cellular immune responses, playing a critical role. Some clues to the underlying mechanisms of rapid disease progression can be gleaned from some of its unique virological and immunologic features. The predictors of rapid progression in SIV-infected macaques are high viremia, irreversible and severe loss of memory $CD4^+$ T cells, and transient immune responses against SIV. There is an undefined threshold in terms of the level of viremia and the early loss of memory $CD4^+$ T cells that is essential for precipitating the failure to maintain immune responses. Studies of these animals suggest that the pathogenesis of rapid disease lacks many of the components of conventional disease progression, such as immune activation-induced cell death and regenerative failure. Therefore, these animals may not be the most accurate model for AIDS in humans. However, they provide the opportunity to examine direct effects of the virus uncomplicated by the effects of virus-induced immune responses.

Acknowledgments

The authors wish to thank those people in the laboratory whose work is summarized here, particularly Charles R. Brown and Takeo Kuwata. We also thank Louis Picker for allowing us to use virus load and survival data from his cohort of SIV-mac239-inoculated macaques.

References

Alexander, L., Illyinskii, P. O., Lang, S. M., Means, R. E., Lifson, J., Mansfield, K., and Desrosiers, R. C. (2003). Determinants of increased replicative capacity of serially passaged simian immunodeficiency virus with nef deleted in rhesus monkeys. *J. Virol.* **77**(12), 6823–6835.

Anderson, M. G., Hauer, D., Sharma, D. P., Joag, S. V., Narayan, O., Zink, M. C., and Clements, J. E. (1993). Analysis of envelope changes acquired by SIVmac239 during neuroadaption in rhesus macaques. *Virology* **195**(2), 616–626.

Anthony, K. B., Yoder, C., Metcalf, J. A., DerSimonian, R., Orenstein, J. M., Stevens, R. A., Falloon, J., Polis, M. A., Lane, H. C., and Sereti, I. (2003). Incomplete CD4 T cell recovery in HIV-1 infection after 12 months of highly active antiretroviral therapy is associated with ongoing increased CD4 T cell activation and turnover. *J. Acquir. Immune Defic. Syndr.* **33**(2), 125–133.

Apetrei, C., Robertson, D. L., and Marx, P. A. (2004). The history of SIVS and AIDS: Epidemiology, phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa. *Front. Biosci.* **9**, 225–254.

Barouch, D. H., Kunstman, J., Glowczwskie, J., Kunstman, K. J., Egan, M. A., Peyerl, F. W., Santra, S., Kuroda, M. J., Schmitz, J. E., Beaudry, K., Krivulka, G. R., Lifton, M. A., *et al.* (2003). Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. *J. Virol.* **77**(13), 7367–7375.

Baskin, G. B., Murphey-Corb, M., Watson, E. A., and Martin, L. N. (1988). Necropsy findings in rhesus monkeys experimentally infected with cultured simian immunodeficiency virus (SIV)/delta. *Vet. Pathol.* **25**(6), 456–467.

Baskin, G. B., Murphey, C. M., Roberts, E. D., Didier, P. J., and Martin, L. N. (1992). Correlates of SIV encephalitis in rhesus monkeys. *J. Med. Primatol.* **21**(2–3), 59–63.

Berger, E. A., Murphy, P. M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annu. Rev. Immunol.* **17**, 657–700.

Bhattacharya, J., Peters, P. J., and Clapham, P. R. (2003). CD4-independent infection of HIV and SIV: Implications for envelope conformation and cell tropism *in vivo*. *AIDS* **17**, (Suppl 4), S35–S43.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T., and Douek, D. C. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* **200**(6), 749–759.

Brown, C. R., Czapiga, M., Kabat, J., Dang, Q., Ourmanov, I., Nishimura, Y., Martin, M. A., and Hirsch, V. M. (2007). Unique pathology in simian immunodeficiency virus infected rapid progressor macaques is consistent with distinct pathogenesis from classical AIDS. *J. Virol.* **81**(11), 5594–5606.

Campbell, B. J., and Hirsch, V. M. (1994). Extensive envelope heterogeneity of simian immunodeficiency virus in tissues from infected macaques. *J. Virol.* **68**(5), 3129–3137.

Cao, Y., Qin, L., Zhang, L., Safrit, J., and Ho, D. D. (1996). Characterization of long-term survivors of human immunodeficiency virus type 1 infection. *Immunol. Lett.* **51**(1–2), 7–13.

Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K., and O'Brien, S. J. (1999). HLA and HIV-1: Heterozygote advantage and B*35-Cw*04 disadvantage. *Science* **283**, 1748–1752.

Chakrabarti, L. A. (2004). The paradox of simian immunodeficiency virus infection in sooty mangabeys: Active viral replication without disease progression. *Front. Biosci.* **9**, 521–539.

Chalifoux, L. V., Ringler, D. J., King, N. W., Sehgal, P. K., Desrosiers, R. C., Daniel, M. D., and Letvin, N. L. (1987). Lymphadenopathy in macaques experimentally infected with the simian immunodeficiency virus (SIV). *Am. J. Pathol.* **128**(1), 104–110.

Chen, Z., Telfier, P., Gettie, A., Reed, P., Zhang, L., Ho, D. D., and Marx, P. A. (1996). Genetic characterization of new West African simian immunodeficiency virus SIVsm: Geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. *J. Virol.* **70**(6), 3617–3627.

Chen, Z., Zhou, P., Ho, D. D., Landau, N. R., and Marx, P. A. (1997). Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. *J. Virol.* **71**(4), 2705–2714.

Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., Kanki, P. J., Essex, M., and Desrosiers, R. C. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**(4704), 1201–1204.

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**(5090), 1938–1941.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., *et al.* (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**(5283), 1856–1862.

Deeks, S. G., and Walker, B. D. (2004). The immune response to AIDS virus infection: Good, bad, or both? *J. Clin. Invest.* **113**(6), 808–810.

Deeks, S. G., Kitchen, C. M., Liu, L., Guo, H., Gascon, R., Narvaez, A. B., Hunt, P., Martin, J. N., Kahn, J. O., Levy, J., McGrath, M. S., and Hecht, F. M. (2004). Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* **104**(4), 942–947.

Dehghani, H., Puffer, B. A., Doms, R. W., and Hirsch, V. M. (2003). Unique pattern of convergent envelope evolution in simian immunodeficiency virus-infected rapid progressor macaques: Association with CD4-independent usage of CCR5. *J. Virol.* **77**(11), 6405–6418.

Desrosiers, R. C. (1990). The simian immunodeficiency viruses. *Annu. Rev. Immunol.* **8**, 557–578.

Desrosiers, R. C., Lifson, J. D., Gibbs, J. S., Czajak, S. C., Howe, A. Y., Arthur, L. O., and Johnson, R. P. (1998). Identification of highly attenuated mutants of simian immunodeficiency virus. *J. Virol.* **72**(2), 1431–1437.

Dewhurst, S., Embretson, J. E., Fultz, P. N., and Mullins, J. I. (1992). Molecular clones from a non-acutely pathogenic derivative of SIVsmmPBj14: Characterization and comparison to acutely pathogenic clones. *AIDS Res. Hum. Retroviruses* **8**(6), 1179–1187.

Douek, D. C. (2003). Disrupting T-cell homeostasis: How HIV-1 infection causes disease. *AIDS Rev.* **5**(3), 172–177.

Douek, D. C., Picker, L. J., and Koup, R. A. (2003). T cell dynamics in HIV-1 infection. *Annu. Rev. Immunol.* **21**, 265–304.

Du, Z., Lang, S. M., Sasseville, V. G., Lackner, A. A., Ilyinskii, P. O., Daniel, M. D., Jung, J. U., and Desrosiers, R. C. (1995). Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell* **82**(4), 665–674.

Edinger, A. L., Amedee, A., Miller, K., Doranz, B. J., Endres, M., Sharron, M., Samson, M., Lu, Z. H., Clements, J. E., Murphey, C. M., Peiper, S. C., Parmentier, M., *et al.* (1997a). Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. *Proc. Natl. Acad. Sci. USA* **94**(8), 4005–4010.

Edinger, A. L., Mankowski, J. L., Doranz, B. J., Margulies, L., Lee, B., Rucker, J., Sharron, M., Hoffman, T. L., Berson, J. F., Zink, M. C., Hirsch, V. M., Clements, J. E., *et al.* (1997b). CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. *Proc. Natl. Acad. Sci. USA* **94**, 14742–14747.

Edmonson, P., Murphey-Corb, M., Martin, L. N., Delahunty, C., Heeney, J., Kornfeld, H., Donahue, P. R., Learn, G. H., Hood, L., and Mullins, J. I. (1998). Evolution of a simian immunodeficiency virus pathogen. *J Virol.* **72**, 405–414.

Evans, D. T., Knapp, L. A., Jing, P., Mitchen, J. L., Dykhuizen, M., Montefiori, D. C., Pauza, C. D., and Watkins, D. I. (1999). Rapid and slow progressors differ by a single MHC class I haplotype in a family of MHC-defined rhesus macaques infected with SIV. *Immunol. Lett.* **66**(1–3), 53–59.

Finkel, T. H., and Banda, N. K. (1994). Indirect mechanisms of HIV pathogenesis: How does HIV kill T cells? *Curr. Opin. Immunol.* **6**(4), 605–615.

Friedrich, T. C., McDermott, A. B., Reynolds, M. R., Piaskowski, S., Fuenger, S., De Souza, I. P., Rudersdorf, R., Cullen, C., Yant, L. J., Vojnov, L., Stephany, J., Martin, S., *et al.* (2004). Consequences of cytotoxic T-lymphocyte escape: Common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. *J. Virol.* **78**(18), 10064–10073.

Frumkin, L. R., Agy, M. B., Coombs, R. W., Panther, L., Morton, W. R., Koehler, J., Florey, M. J., Dragavon, J., Schmidt, A., Katze, M. G., and Corey, L. (1993). Acute infection of Macaca nemestrina by human immunodeficiency virus type 1. *Virology* **195**(2), 422–431.

Fultz, P. N., and Zack, P. M. (1994). Unique lentivirus–host interactions: SIVsmmPBj14 infection of macaques. *Virus Res.* **32**(2), 205–225.

Fultz, P. N., McClure, H. M., Anderson, D. C., and Switzer, W. M. (1989). Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). *AIDS Res. Hum. Retroviruses* **5**(4), 397–409.

Garland, F. C., Garland, C. F., Gorham, E. D., and Brodine, S. K. (1996). Western blot banding patterns of HIV rapid progressors in the U.S. Navy Seropositive Cohort: Implications for vaccine development. Navy Retroviral Working Group. *Ann. Epidemiol.* **6**(4), 341–347.

Giorgi, J. V., Hultin, L. E., McKeating, J. A., Johnson, T. D., Owens, B., Jacobson, L. P., Shih, R., Lewis, J., Wiley, D. J., Phair, J. P., Wolinsky, S. M., and Detels, R. (1999). Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J. Infect. Dis.* **179**(4), 859–870.

Hahn, B. H., Shaw, G. M., De Cock, K. M., and Sharp, P. M. (2000). AIDS as a zoonosis: Scientific and public health implications. *Science* **287**(5453), 607–614.

Hazenberg, M. D., Hamann, D., Schuitemaker, H., and Miedema, F. (2000). T cell depletion in HIV-1 infection: How CD4+ T cells go out of stock. *Nat. Immunol.* **1**(4), 285–289.

Hirsch, V. M., and Johnson, P. R. (1994). Pathogenic diversity of simian immunodeficiency viruses. *Virus Res.* **32**(2), 183–203.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H., and Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**(6223), 389–392.

Hirsch, V. M., Zack, P. M., Vogel, A. P., and Johnson, P. R. (1991). Simian immunodeficiency virus infection of macaques: End-stage disease is characterized by widespread distribution of proviral DNA in tissues. *J. Infect. Dis.* **163**(5), 976–988.

Hirsch, V. M., Goldstein, S., Hynes, N. A., Elkins, W. R., London, W. T., Zack, P. M., Montefiori, D., and Johnson, P. R. (1994a). Prolonged clinical latency and survival of macaques given a whole inactivated simian immunodeficiency virus vaccine. *J. Infect. Dis.* **170**(1), 51–59.

Hirsch, V. M., Martin, J. E., Dapolito, G., Elkins, W. R., London, W. T., Goldstein, S., and Johnson, P. R. (1994b). Spontaneous substitutions in the vicinity of the V3 analog affect cell tropism and pathogenicity of simian immunodeficiency virus. *J. Virol.* **68**(4), 2649–2661.

Hirsch, V. M., Dapolito, G., Goeken, R., and Campbell, B. J. (1995). Phylogeny and natural history of the primate lentiviruses, SIV and HIV. *Curr. Opin. Genet. Dev.* **5**(6), 798–806.

Hirsch, V. M., Fuerst, T. R., Sutter, G., Carroll, M. W., Yang, L. C., Goldstein, S., Piatak, M. J., Elkins, W. R., Alvord, W. D., Montefiori, D. C., Moss, B., and Lifson, J. D. (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: Effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* **70**, 3741–3752.

Hirsch, V. M., Sharkey, M. E., Brown, C. R., Brichacek, B., Goldstein, S., Wakefield, J., Byrum, R., Elkins, W. R., Hahn, B. H., Lifson, J. D., and Stevenson, M. (1998). Vpx is required for dissemination and pathogenesis of SIV(SM) PBj: Evidence of macrophage-dependent viral amplification. *Nat. Med.* **4**(12), 1401–1408.

Hirsch, V. M., Santra, S., Goldstein, S., Plishka, R., Buckler-White, A., Seth, A., Ourmanov, I., Brown, C. R., Engle, R., Montefiori, D., Glowczwskie, J., Kunstman, K., *et al.* (2004). Immune failure in the absence of profound CD4+ T-lymphocyte depletion in simian immunodeficiency virus-infected rapid progressor macaques. *J. Virol.* **78**(1), 275–284.

Holterman, L., Niphuis, H., ten Haaft, P. J., Goudsmit, J., Baskin, G., and Heeney, J. L. (1999). Specific passage of simian immunodeficiency virus from end-stage disease results in accelerated progression to AIDS in rhesus macaques. *J. Gen. Virol.* **80**(Pt 12), 3089–3097.

Itescu, S., Mathur-Wagh, U., Skovron, M. L., Brancato, L. J., Marmor, M., Zeleniuch-Jacquotte, A., and Winchester, R. (1992). HLA-B35 is associated with accelerated progression to AIDS. *J. Acquir. Immune Defic. Syndr.* **5**(1), 37–45.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., *et al.* (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* **189**(6), 991–998.

Joag, S. V., Stephens, E. B., Adams, R. J., Foresman, L., and Narayan, O. (1994). Pathogenesis of SIVmac infection in Chinese and Indian rhesus macaques: Effects of splenectomy on virus burden. *Virology* **200**(2), 436–446.

Johnson, P. R., and Hirsch, V. M. (1992). Genetic variation of simian immunodeficiency viruses in nonhuman primates. *AIDS Res. Hum. Retroviruses* **8**(3), 367–372.

Kaslow, R. A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A. J., Goedert, J. J., Winkler, C., O'Brien, S. J., Rinaldo, C., Detels, R., Blattner, W., *et al.* (1996). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* **2**(4), 405–411.

Kaur, A., Daniel, M. D., Hempel, D., Lee-Parritz, D., Hirsch, M. S., and Johnson, R. P. (1996). Cytotoxic T-lymphocyte responses to cytomegalovirus in normal and simian immunodeficiency virus-infected rhesus macaques. *J. Virol.* **70**(11), 7725–7733.

Kaur, A., Hale, C. L., Noren, B., Kassis, N., Simon, M. A., and Johnson, R. P. (2002). Decreased frequency of cytomegalovirus (CMV)-specific CD4+ T lymphocytes in simian immunodeficiency virus-infected rhesus macaques: Inverse relationship with CMV viremia. *J. Virol.* **76**(8), 3646–3658.

Kaur, A., Kassis, N., Hale, C. L., Simon, M., Elliott, M., Gomez-Yafal, A., Lifson, J. D., Desrosiers, R. C., Wang, F., Barry, P., Mach, M., and Johnson, R. P. (2003). Direct relationship between suppression of virus-specific immunity and emergence of cytomegalovirus disease in simian AIDS. *J. Virol.* **77**(10), 5749–5758.

Kestler, H. W., 3rd, Li, Y., Naidu, Y. M., Butler, C. V., Ochs, M. F., Jaenel, G., King, N. W., Daniel, M. D., and Desrosiers, R. C. (1988). Comparison of simian immunodeficiency virus isolates. *Nature.* **331**, 619–622.

Kestler, H. W., 3rd, Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**(4), 651–662.

Kimata, J. T., Kuller, L., Anderson, D. B., Dailey, P., and Overbaugh, J. (1999). Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. *Nat. Med.* **5**(5), 535–541.

Kodama, T., Mori, K., Kawahara, T., Ringler, D. J., and Desrosiers, R. C. (1993). Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. *J. Virol.* **67**(11), 6522–6534.

Koot, M., Keet, I. P., Vos, A. H., de Goede, R. E., Roos, M. T., Coutinho, R. A., Miedema, F., Schellekens, P. T., and Tersmette, M. (1993). Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Ann. Intern. Med.* **118**(9), 681–688.

Koup, R. A. (1994). Virus escape from CTL recognition. *J. Exp. Med.* **180**(3), 779–782.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**(7), 4650–4655.

Kovacs, J. A., Lempicki, R. A., Sidorov, I. A., Adelsberger, J. W., Herpin, B., Metcalf, J. A., Sereti, I., Polis, M. A., Davey, R. T., Tavel, J., Falloon, J., Stevens, R., *et al.* (2001). Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. *J. Exp. Med.* **194**(12), 1731–1741.

Kunstman, K. J., Puffer, B., Korber, B. T., Kuiken, C., Smith, U. R., Kunstman, J., Stanton, J., Agy, M., Shibata, R., Yoder, A. D., Pillai, S., Doms, R. W., *et al.* (2003). Structure and function of CC-chemokine receptor 5 homologues derived from representative primate species and subspecies of the taxonomic suborders Prosimii and Anthropoidea. *J. Virol.* **77**(22), 12310–12318.

Kuwata, T., Dehghani, H., Brown, C. R., Plishka, R., Buckler-White, A., Igarashi, T., Mattapallil, J., Roederer, M., and Hirsch, V. M. (2006). Infectious molecular clones from a simian immunodeficiency virus-infected rapid-progressor (RP) macaque: Evidence of differential selection of RP-specific envelope mutations *in vitro* and *in vivo*. *J. Virol.* **80**(3), 1463–1475.

Lackner, A. A., and Veazey, R. S. (2007). Current concepts in AIDS pathogenesis: Insights from the SIV/macaque model. *Annu. Rev. Med.* **58**, 461–476.

Letvin, N. L., and King, N. W. (1990). Immunologic and pathologic manifestations of the infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J. Acquir. Immune Defic. Syndr.* **3**(11), 1023–1040.

Letvin, N. L., Daniel, M. D., Seghal, P. K., Desrosiers, R. C., Hunt, R. D., Waldron, L. M., MacKey, J. J., Schmidt, D. K., Chalifoux, L. V., and King, N. W. (1985). Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* **230**, 71–73.

Letvin, N. L., Mascola, J. R., Sun, Y., Gorgone, D. A., Buzby, A. P., Xu, L., Yang, Z. Y., Chakrabarti, B., Rao, S. S., Schmitz, J. E., Montefiori, D. C., Barker, B. R., *et al*. (2006). Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* **312**(5779), 1530–1533.

Lewis, M. G., Zack, P. M., Elkins, W. R., and Jahrling, P. B. (1992). Infection of rhesus and cynomolgus macaques with a rapidly fatal SIV (SIVSMM/PBj) isolate from sooty mangabeys. *AIDS Res. Hum. Retroviruses* **8**(9), 1631–1639.

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J., and Haase, A. T. (2005). Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* **434**(7037), 1148–1152.

Lifson, J. D., Nowak, M. A., Goldstein, S., Rossio, J. L., Kinter, A., Vasquez, G., Wiltrout, T. A., Brown, C., Schneider, D., Wahl, L., Lloyd, A. L., Williams, J., *et al*. (1997). The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. *J. Virol.* **71**(12), 9508–9514.

Ling, B., Veazey, R. S., Luckay, A., Penedo, C., Xu, K., Lifson, J. D., and Marx, P. A. (2002). SIV(mac) pathogenesis in rhesus macaques of Chinese and Indian origin compared with primary HIV infections in humans. *AIDS* **16**(11), 1489–1496.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**(3), 367–377.

Luciw, P. A., Shaw, K. E., Unger, R. E., Planelles, V., Stout, M. W., Lackner, J. E., Pratt-Lowe, E., Leung, N. J., Banapour, B., and Marthas, M. L. (1992). Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). *AIDS Res. Hum. Retroviruses*. **8**, 395–402. Review.

Marthas, M. L., Ramos, R. A., Lohman, B. L., Van Rompay, K. K., Unger, R. E., Miller, C. J., Banapour, B., Pedersen, N. C., and Luciw, P. A. (1993). Viral determinants of simian immunodeficiency virus (SIV) virulence in rhesus macaques assessed by using attenuated and pathogenic molecular clones of SIVmac. *J Virol.* **67**, 6047–6055.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., and Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**(7037), 1093–1097.

Mattapallil, J. J., Douek, D. C., Buckler-White, A., Montefiori, D., Letvin, N. L., Nabel, G. J., and Roederer, M. (2006). Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J. Exp. Med.* **203**(6), 1533–1541.

McClure, H. M., Anderson, D. C., Fultz, P. N., Ansari, A. A., Lockwood, E., and Brodie, A. (1989). Spectrum of disease in macaque monkeys chronically infected with SIV/SMM. *Vet. Immunol. Immunopathol.* **21**(1), 13–24.

Means, R. E., Matthews, T., Hoxie, J. A., Malim, M. H., Kodama, T., and Desrosiers, R. C. (2001). Ability of the V3 loop of simian immunodeficiency virus to serve as a target for antibody-mediated neutralization: Correlation of neutralization sensitivity, growth in macrophages, and decreased dependence on CD4. *J. Virol.* **75**(8), 3903–3915.

Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A., and Kingsley, L. A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* **272**(5265), 1167–1170.

Mellors, J. W., Munoz, A., Giorgi, J. V., Margolick, J. B., Tassoni, C. J., Gupta, P., Kingsley, L. A., Todd, J. A., Saah, A. J., Detels, R., Phair, J. P., and Rinaldo, C. R., Jr. (1997). Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann. Intern. Med.* **126**(12), 946–954.

Michael, N. L., Brown, A. E., Voigt, R. F., Frankel, S. S., Mascola, J. R., Brothers, K. S., Louder, M., Birx, D. L., and Cassol, S. A. (1997). Rapid disease progression without

seroconversion following primary human immunodeficiency virus type 1 infection–evidence for highly susceptible human hosts. *J. Infect. Dis.* **175**(6), 1352–1359.

Migueles, S. A., and Connors, M. (2002). The role of CD4(+) and CD8(+) T cells in controlling HIV infection. *Curr. Infect. Dis. Rep.* **4**(5), 461–467.

Miller, M. D., Yamamoto, H., Hughes, A. L., Watkins, D. I., and Letvin, N. L. (1991). Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J. Immunol.* **147**(1), 320–329.

Mohri, H., Perelson, A. S., Tung, K., Ribeiro, R. M., Ramratnam, B., Markowitz, M., Kost, R., Hurley, A., Weinberger, L., Cesar, D., Hellerstein, M. K., and Ho, D. D. (2001). Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J. Exp. Med.* **194**(9), 1277–1287.

Montagnier, L., Brenner, C., Chamaret, S., Guetard, D., Blanchard, A., de Saint Martin, J., Poveda, J. D., Pialoux, G., and Gougeon, M. L. (1997). Human immunodeficiency virus infection and AIDS in a person with negative serology. *J. Infect. Dis.* **175**(4), 955–959.

Mori, K., Ringler, D. J., Kodama, T., and Desrosiers, R. C. (1992). Complex determinants of macrophage tropism in env of simian immunodeficiency virus. *J. Virol.* **66**(4), 2067–2075.

Mori, K., Rosenzweig, M., and Desrosiers, R. C. (2000). Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. *J. Virol.* **74**(22), 10852–10859.

Moss, A. R., and Bacchetti, P. (1989). Natural history of HIV infection. *AIDS* **3**(2), 55–61.

Muhl, T., Krawczak, M., Ten Haaft, P., Hunsmann, G., and Sauermann, U. (2002). MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. *J. Immunol.* **169**(6), 3438–3446.

Nishimura, Y., Igarashi, T., Donau, O. K., Buckler-White, A., Buckler, C., Lafont, B. A., Goeken, R. M., Goldstein, S., Hirsch, V. M., and Martin, M. A. (2004). Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc. Natl. Acad. Sci. USA* **101**(33), 12324–12329.

Nishimura, Y., Igarashi, T., Buckler-White, A., Buckler, C., Imamichi, H., Goeken, R. M., Lee, W. R., Lafont, B. A., Byrum, R., Lane, H. C., Hirsch, V. M., and Martin, M. A. (2007). Loss of naive cells accompanies memory CD4+ T-cell depletion during long-term progression to AIDS in Simian immunodeficiency virus-infected macaques. *J. Virol.* **81**(2), 893–902.

Novembre, F. J., Johnson, P. R., Lewis, M. G., Anderson, D. C., Klumpp, S., McClure, H. M., and Hirsch, V. M. (1993). Multiple viral determinants contribute to pathogenicity of the acutely lethal simian immunodeficiency virus SIVsmmPBj variant. *J. Virol.* **67**(5), 2466–2474.

Novembre, F. J., Saucier, M. M., Hirsch, V. M., Johnson, P. R., and McClure, H. M. (1994). Viral genetic determinants in SIVsmmPBj pathogenesis. *J. Med. Primatol.* **23**(2–3), 136–145.

Novembre, F. J., De Rosayro, J., O'Neil, S. P., Anderson, D. C., Klumpp, S. A., and McClure, H. M. (1998). Isolation and characterization of a neuropathogenic simian immunodeficiency virus derived from a sooty mangabey. *J. Virol.* **72**(11), 8841–8851.

O'Connor, D. H., Mothe, B. R., Weinfurter, J. T., Fuenger, S., Rehrauer, W. M., Jing, P., Rudersdorf, R. R., Liebl, M. E., Krebs, K., Vasquez, J., Dodds, E., Loffredo, J., *et al.* (2003). Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. *J. Virol.* **77**(16), 9029–9040.

O'Neil, S. P., Suwyn, C., Anderson, D. C., Niedziela, G., Bradley, J., Novembre, F. J., Herndon, J. G., and McClure, H. M. (2004). Correlation of acute humoral response with brain virus burden and survival time in pig-tailed macaques infected with the

neurovirulent simian immunodeficiency virus SIVsmmFGb. *Am. J. Pathol.* **164**(4), 1157–1172.

Orandle, M. S., Williams, K. C., MacLean, A. G., Westmoreland, S. V., and Lackner, A. A. (2001). Macaques with rapid disease progression and simian immunodeficiency virus encephalitis have a unique cytokine profile in peripheral lymphoid tissues. *J. Virol.* **75**(9), 4448–4452.

Otting, N., Heijmans, C. M., Noort, R. C., de Groot, N. G., Doxiadis, G. G., van Rood, J. J., Watkins, D. I., and Bontrop, R. E. (2005). Unparalleled complexity of the MHC class I region in rhesus macaques. *Proc. Natl. Acad. Sci. USA* **102**(5), 1626–1631.

Ourmanov, I., Brown, C. R., Moss, B., Carroll, M., Wyatt, L., Pletneva, L., Goldstein, S., Venzon, D., and Hirsch, V. M. (2000). Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *J. Virol.* **74**(6), 2740–2751.

Overbaugh, J., Rudensey, L. M., Papenhausen, M. D., Benveniste, R. E., and Morton, W. R. (1991). Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. *J. Virol.* **65**(12), 7025–7031.

Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O. J., Demarest, J. F., Montefiori, D., Orenstein, J. M., Fox, C., Schrager, L. K., Margolick, J. B., Buchbinder, S., *et al.* (1995). Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N. Engl. J. Med.* **332**(4), 209–216.

Parham, P., and Ohta, T. (1996). Population biology of antigen presentation by MHC class I molecules. *Science* **272**(5258), 67–74.

Piatak, M., Jr., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K. C., Hahn, B. H., Shaw, G. M., and Lifson, J. D. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259**(5102), 1749–1754.

Picker, L. J. (2006). Immunopathogenesis of acute AIDS virus infection. *Curr. Opin. Immunol.* **18**(4), 399–405.

Picker, L. J., Hagen, S. I., Lum, R., Reed-Inderbitzin, E. F., Daly, L. M., Sylwester, A. W., Walker, J. M., Siess, D. C., Piatak, M., Jr., Wang, C., Allison, D. B., Maino, V. C., *et al.* (2004). Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. *J. Exp. Med.* **200**(10), 1299–1314.

Pitcher, C. J., Hagen, S. I., Walker, J. M., Lum, R., Mitchell, B. L., Maino, V. C., Axthelm, M. K., and Picker, L. J. (2002). Development and homeostasis of T cell memory in rhesus macaque. *J. Immunol.* **168**(1), 29–43.

Puffer, B. A., Pohlmann, S., Edinger, A. L., Carlin, D., Sanchez, M. D., Reitter, J., Watry, D. D., Fox, H. S., Desrosiers, R. C., and Doms, R. W. (2002). CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. *J. Virol.* **76**(6), 2595–2605.

Ringler, D. J., Hunt, R. D., Desrosiers, R. C., Daniel, M. D., Chalifoux, L. V., and King, N. W. (1988). Simian immunodeficiency virus-induced meningoencephalitis: Natural history and retrospective study. *Ann. Neurol.* **23**(Suppl.), S101–S107.

Roberts, E. S., Zandonatti, M. A., Watry, D. D., Madden, L. J., Henriksen, S. J., Taffe, M. A., and Fox, H. S. (2003). Induction of pathogenic sets of genes in macrophages and neurons in NeuroAIDS. *Am. J. Pathol.* **162**(6), 2041–2057.

Rodriguez, B., Sethi, A. K., Cheruvu, V. K., Mackay, W., Bosch, R. J., Kitahata, M., Boswell, S. L., Mathews, W. C., Bangsberg, D. R., Martin, J., Whalen, C. C., Sieg, S., *et al.* (2006). Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection. *JAMA* **296**(12), 1498–1506.

Roeth, J. F., and Collins, K. L. (2006). Human immunodeficiency virus type 1 Nef: Adapting to intracellular trafficking pathways. *Microbiol. Mol. Biol. Rev.* **70**(2), 548–563.

Rosenberg, Y. J., White, B. D., Papermaster, S. F., Zack, P., Jarling, P. B., Eddy, G. A., Burke, D. S., and Lewis, M. G. (1991). Variation in T-lymphocyte activation and susceptibility to SIVPBj-14-induced acute death in macaques. *J. Med. Primatol.* **20**(4), 206–210.

Rudensey, L. M., Kimata, J. T., Benveniste, R. E., and Overbaugh, J. (1995). Progression to AIDS in macaques is associated with changes in the replication, tropism, and cytopathic properties of the simian immunodeficiency virus variant population. *Virology* **207**(2), 528–542.

Ryzhova, E., Whitbeck, J. C., Canziani, G., Westmoreland, S. V., Cohen, G. H., Eisenberg, R. J., Lackner, A., and Gonzalez-Scarano, F. (2002). Rapid progression to simian AIDS can be accompanied by selection of CD4-independent gp120 variants with impaired ability to bind CD4. *J. Virol.* **76**(15), 7903–7909.

Santiago, M. L., Rodenburg, C. M., Kamenya, S., Bibollet-Ruche, F., Gao, F., Bailes, E., Meleth, S., Soong, S. J., Kilby, J. M., Moldoveanu, Z., Fahey, B., Muller, M. N., *et al.* (2002). SIVcpz in wild chimpanzees. *Science* **295**(5554), 465.

Sauermann, U., Krawczak, M., Hunsmann, G., and Stahl-Hennig, C. (1997). Identification of Mhc-Mamu-DQB1 allele combinations associated with rapid disease progression in rhesus macaques infected with simian immunodeficiency virus. *AIDS* **11**(9), 1196–1198.

Sauermann, U., Stahl-Hennig, C., Stolte, N., Muhl, T., Krawczak, M., Spring, M., Fuchs, D., Kaup, F. J., Hunsmann, G., and Sopper, S. (2000). Homozygosity for a conserved Mhc class II DQ-DRB haplotype is associated with rapid disease progression in simian immunodeficiency virus-infected macaques: Results from a prospective study. *J. Infect. Dis.* **182**(3), 716–724.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., *et al.* (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**(5403), 857–860.

Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., de Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F., and Tersmette, M. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: Progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J. Virol.* **66**(3), 1354–1360.

Silvestri, G. (2005). Naturally SIV-infected sooty mangabeys: Are we closer to understanding why they do not develop AIDS? *J. Med. Primatol.* **34**(5–6), 243–252.

Silvestri, G., Sodora, D. L., Koup, R. A., Paiardini, M., O'Neil, S. P., McClure, H. M., Staprans, S. I., and Feinberg, M. B. (2003). Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* **18**(3), 441–452.

Simon, M. A., Chalifoux, L. V., and Ringler, D. J. (1992). Pathologic features of SIV-induced disease and the association of macrophage infection with disease evolution. *AIDS Res. Hum. Retroviruses* **8**(3), 327–337.

Simon, M. A., Brodie, S. J., Sasseville, V. G., Chalifoux, L. V., Desrosiers, R. C., and Ringler, D. J. (1994). Immunopathogenesis of SIVmac. *Virus Res.* **32**(2), 227–251.

Staprans, S. I., Dailey, P. J., Rosenthal, A., Horton, C., Grant, R. M., Lerche, N., and Feinberg, M. B. (1999). Simian immunodeficiency virus disease course is predicted by the extent of virus replication during primary infection. *J. Virol.* **73**(6), 4829–4839.

Tang, J., Costello, C., Keet, I. P., Rivers, C., Leblanc, S., Karita, E., Allen, S., and Kaslow, R. A. (1999). HLA class I homozygosity accelerates disease progression in human immunodeficiency virus type 1 infection. *AIDS Res. Hum. Retroviruses* **15**(4), 317–324.

Trichel, A. M., Rajakumar, P. A., and Murphey-Corb, M. (2002). Species-specific variation in SIV disease progression between Chinese and Indian subspecies of rhesus macaque. *J. Med. Primatol.* **31**(4–5), 171–178.

VandeWoude, S., and Apetrei, C. (2006). Going wild: Lessons from naturally occurring T-lymphotropic lentiviruses. *Clin. Microbiol. Rev.* **19**(4), 728–762.

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C., and Lackner, A. A. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**(5362), 427–431.

Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E., and Lackner, A. A. (2000). Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. *J. Virol.* **74**(23), 11001–11007.

Watson, A., Ranchalis, J., Travis, B., McClure, J., Sutton, W., Johnson, P. R., Hu, S. L., and Haigwood, N. L. (1997). Plasma viremia in macaques infected with simian immunodeficiency virus: Plasma viral load early in infection predicts survival. *J. Virol.* **71**(1), 284–290.

Westmoreland, S. V., Halpern, E., and Lackner, A. A. (1998). Simian immunodeficiency virus encephalitis in rhesus macaques is associated with rapid disease progression. *J. Neurovirol.* **4**(3), 260–268.

Wyand, M. S., Ringler, D. J., Naidu, Y. M., Mattmuller, M., Chalifoux, L. V., Sehgal, P. K., Daniel, M. D., Desrosiers, R. C., and King, N. W. (1989). Cellular localization of simian immunodeficiency virus in lymphoid tissues. II. In situ hybridization. *Am. J. Pathol.* **134**(2), 385–393.

Zhang, J. Y., Martin, L. N., Watson, E. A., Montelaro, R. C., West, M., Epstein, L., and Murphey-Corb, M. (1988). Simian immunodeficiency virus/delta-induced immunodeficiency disease in rhesus monkeys: Relation of antibody response and antigenemia. *J. Infect. Dis.* **158**(6), 1277–1286.

Zhang, L., He, T., Huang, Y., Chen, Z., Guo, Y., Wu, S., Kunstman, K. J., Brown, R. C., Phair, J. P., Neumann, A. U., Ho, D. D., and Wolinsky, S. M. (1998). Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. *J. Virol.* **72**(11), 9307–9312.

Zhang, Z. Q., Casimiro, D. R., Schleif, W. A., Chen, M., Citron, M., Davies, M. E., Burns, J., Liang, X., Fu, T. M., Handt, L., Emini, E. A., and Shiver, J. W. (2007). Early depletion of proliferating B cells of germinal center in rapidly progressive simian immunodeficiency virus infection. *Virology* **361**(2), 455–464.

Zink, M. C., Amedee, A. M., Mankowski, J. L., Craig, L., Didier, P., Carter, D. L., Munoz, A., Murphey-Corb, M., and Clements, J. E. (1997). Pathogenesis of SIV encephalitis. Selection and replication of neurovirulent SIV. *Am. J. Pathol.* **151**(3), 793–803.

Zink, M. C., Suryanarayana, K., Mankowski, J. L., Shen, A., Piatak, M., Jr., Spelman, J. P., Carter, D. L., Adams, R. J., Lifson, J. D., and Clements, J. E. (1999). High viral load in the cerebrospinal fluid and brain correlates with severity of simian immunodeficiency virus encephalitis. *J. Virol.* **73**(12), 10480–10488.

Viet Hoang, Elizabeth Withers-Ward, and David Camerini

Center for Virus Research, University of California, Irvine, California 92697;
Center for Immunology, University of California, Irvine, California 92697; and
Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

Nonprimate Models of HIV-1 Infection and Pathogenesis

I. Chapter Overview

Nonprimate models of *Human immunodeficiency virus 1* (HIV-1) infection and pathogenesis have played an important role in HIV research. These models combine the ease of working with small animals with the power of working with a whole animal model. Several types of nonprimate model have been used. Immunodeficient mice implanted with human lymphoid tissue or cells have proven to be useful systems for numerous studies of HIV-1 replication, cytopathic effects (CPE), and immunity. The recent development of more profoundly immunodeficient mice allows a greater diversity of human

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56013-8

leukocytes to develop and persist in chimeric animals thereby increasing the utility of this approach. Mice, rats, and rabbits expressing human CD4 and other molecules known to be needed for HIV replication have been of limited utility to date since HIV-1 replicates poorly in these animals as currently developed. In contrast, transgenic mice bearing the entire HIV-1 genome or one or more HIV-1 genes have provided insights into the mechanisms of HIV-1 pathogenesis and continue to be useful tools.

II. Introduction

Understanding the pathogenic properties of the HIV-1 during the sequence of molecular events that ultimately result in breakdown of the immune system and AIDS is requisite to identification of potential anti-HIV reagents that limit disease progression. Many key aspects of HIV pathogenesis can only be defined in large animal models that more closely mimic infection in humans. Several African nonhuman primates naturally harbor *Simian immunodeficiency viruses* (SIVs) from which HIV is derived including chimpanzees (SIV-cpz), sooty mangabeys (SIV-sm), and African green monkeys (SIV-agm). These animals have been helpful in evaluating some aspects of HIV infection, although there are major drawbacks. There are significant differences between SIV and HIV and, most importantly, in most cases the simian viruses do not cause development of a progressive immunodeficiency, the hallmark of HIV infection. Moreover, in animals that do develop simian AIDS such as Asian rhesus macaques, some important characteristics of human AIDS, such as coreceptor switching, are not seen. Furthermore, there are prohibitive issues associated with primate studies. They are very expensive, require sophisticated animal facilities, and have a negative impact on the chimpanzee and rhesus populations. Studies of HIV-1 pathogenesis in infected individuals are invaluable but have obvious limitations. In light of these drawbacks, several small animal models designed to recapitulate *in vivo* scenarios of HIV-1 infection have been developed and have been very successful in revealing many aspects of HIV-1 infection and pathogenesis.

Ideally, a small animal model to study HIV-1 infection would allow reconstitution of human cells and tissues that are targets for HIV-1 infection or be permissive for HIV-1 replication in the immune system of the animal. In 1983, a severe combined immunodeficient mouse, C.B.-17 SCID/SCID was identified. SCID mice lack functional mature T and B cells (Bosma *et al.*, 1983; Custer *et al.*, 1985; Dorshkind *et al.*, 1984) due to a mutation in an enzyme responsible for immunoglobulin and T cell receptor (TCR) recombination repair, needed for the functional rearrangement of these genes from the germ line configuration to the expressed versions (Malynn *et al.*, 1988; Schuler *et al.*, 1996). This enables SCID mice to accept xenogeneic tissues

that subsequently form stable grafts. Since the late 1980s, SCID mice have been used successfully to engraft human thymus and liver (Thy/Liv) as well as peripheral blood lymphocytes (PBL) to create the SCID-hu Thy/Liv and human-PBL-SCID (hu-PBL-SCID) models, respectively. SCID-hu Thy/Liv mice have been used to study HIV-1 infection of the thymus while the hu-PBL-SCID model has been used to assess viral phenotype and vaccine efficacy *in vivo*. Blood-forming fetal liver cells, fetal bone, fetal thymus, and fetal lymph nodes have also been engrafted in SCID or nonobese diabetic (NOD)-SCID mice (Christianson *et al*., 1997; Greiner *et al*., 1995; Hesselton *et al*., 1995; McCune *et al*., 1988, 1996; Shultz *et al*., 1995). $Rag^{-/-}$ $\gamma c^{-/-}$ mice, a strain that is more severely immune deficient than SCID or NOD-SCID mice, have been reconstituted with human hematopoietic stem cells (HSC) and infected with HIV-1 (Baenziger *et al*., 2006; Berges *et al*., 2006). HIV-1 transgenic (Tg) mice, humanized mice, rats, and rabbits have also been developed to study HIV infection. In this chapter, we will examine the small animal systems described above, highlight the strengths and limitations of each model, and discuss the results each has contributed to our understanding of HIV-1 infection and pathogenesis.

III. SCID-Hu Thy/Liv Mice

The SCID-hu Thy/Liv mouse is created by coimplantation of human fetal thymus and liver under the kidney capsule of an SCID mouse, resulting in a conjoint Thy/Liv organ. Human fetal liver is included to provide a source of human hematopoietic progenitor cells, while the fetal thymic fragment provides the essential microenvironment for the development of T cells (McCune *et al*., 1988, 1996; Namikawa *et al*., 1986; Touraine, 1983). The Thy/Liv organ implant undergoes significant growth and vascularization, and thymopoiesis continues for 6–12 months or more. Once transplanted, the hematolymphoid system expands rapidly and after three to four months, the graft is ready for infection with HIV-1. Histologically, the SCID-hu Thy/Liv graft is nearly identical to normal human thymus. The microarchitecture or parenchyma of the Thy/Liv graft resembles its human counterpart except that it also contains islets of human fetal liver and mouse MHC class II antigen (MHC-II) positive cells. These murine MHC-II+ cells have a dendritic morphology and are located within the medulla, mid- and deep cortex of the thymic portions of the graft. Normal human $CD4^+$ and $CD8^+$ thymocyte populations are present, including double positive (DP), double negative (DN), CD4 single positive (CD4SP), and CD8 single positive (CD8SP) thymocytes. SCID-hu thymocytes are functionally competent with a normal TCR Vβ repertoire and undergo positive and negative selection that restricts their reactivity and renders them tolerant to self-antigens (Vandekerckhove *et al*., 1992a,b).

After successful implantation, low levels of circulating human T cells can be detected that are phenotypically similar to their counterparts in humans: the CD4/CD8 ratio is 3–4:1, 95% of them express CD3 antigen, 99% have the heterodimeric α/β TCR, and 19% express the peripheral lymph node homing receptor CD62L. Most of the circulating human T cells are quiescent, although about 1% expresses the activation markers, CD25 and CD69. Quiescent T cells can be reactivated after stimulation *in vitro* with CD3 and CD28 antibodies or phytohemagglutinin (PHA) and show an increase in expression of CD25 and CD69.

The initial step in HIV-1 infection is binding of the virion via its surface glycoprotein, gp120, to the host cell surface glycoprotein CD4 and to a coreceptor, usually CCR5 or CXCR4 (Alkhatib *et al.*, 1997, 1996; Bazan *et al.*, 1998; Choe *et al.*, 1998, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Edinger *et al.*, 1998; Farzan *et al.*, 1997; Feng *et al.*, 1996; Frade *et al.*, 1997; He *et al.*, 1997; Horuk *et al.*, 1998; Liao *et al.*, 1997; Loetscher *et al.*, 1997; Owman *et al.*, 1998; Pleskoff *et al.*, 1997; Rucker *et al.*, 1997; Zhang *et al.*, 1998). Isolates of HIV-1 that can enter cells only following interaction with CCR5 are called R5 HIV-1. Similarly, those that can enter only via CXCR4 are called X4, while those that can use both coreceptors are classified as R5X4. HIV-1 isolates capable of using CXCR4 (R5X4 and X4 HIV-1) were formerly called syncytium-inducing (SI) isolates, and their presence in infected individuals has been correlated with more rapid progression to AIDS and death (Connor *et al.*, 1993, 1997). Moreover, R5X4 or X4 HIV-1 clones are more rapidly and completely cytopathic for $CD4^+$ human thymocytes in SCID-hu Thy/Liv mice than R5 HIV-1 (Camerini *et al.*, 2000; Jamieson *et al.*, 1995; Kaneshima *et al.*, 1994). This is likely to be explained by the fact that a much higher percentage of $CD4^+$ thymocytes express CXCR4 than CCR5 (Berkowitz *et al.*, 1998; Kitchen and Zack, 1997, 1999; Taylor *et al.*, 2001). An example of this is shown in Fig. 1; infection with R5 HIV-1 (NSI) had little effect on the proportion of $CD4^+$ thymocytes, compared to mock infection. In contrast, an R5X4 HIV-1 (SI) clone isolated at a later time from the same patient caused a marked depletion of $CD4^+$ thymocytes six weeks postinfection.

HIV-1 infection of the SCID-hu Thy/Liv mouse model accurately reflects the effects of diverse HIV-1 genotypes. In contrast to many tissue culture models, mutation of accessory genes, such as *vpr*, *vif*, *vpu*, *or nef*, slows the replication and moderates the CPE of HIV-1 in the SCID-hu mouse (Aldrovandi and Zack, 1996). Both X4 and R5 HIV-1 replicate and deplete thymocytes in the SCID-hu Thy/Liv mouse model (Jamieson *et al.*, 1995; Scoggins *et al.*, 2000). Moreover, X4 HIV-1 and AIDS-associated R5 HIV-1 clones replicate with better efficiency and exhibit greater CPE than pre-AIDS R5 HIV-1 clones (Camerini *et al.*, 2000; Meissner *et al.*, 2004; Scoggins *et al.*, 2000). The increased affinity for CCR5 of AIDS-associated R5 HIV-1

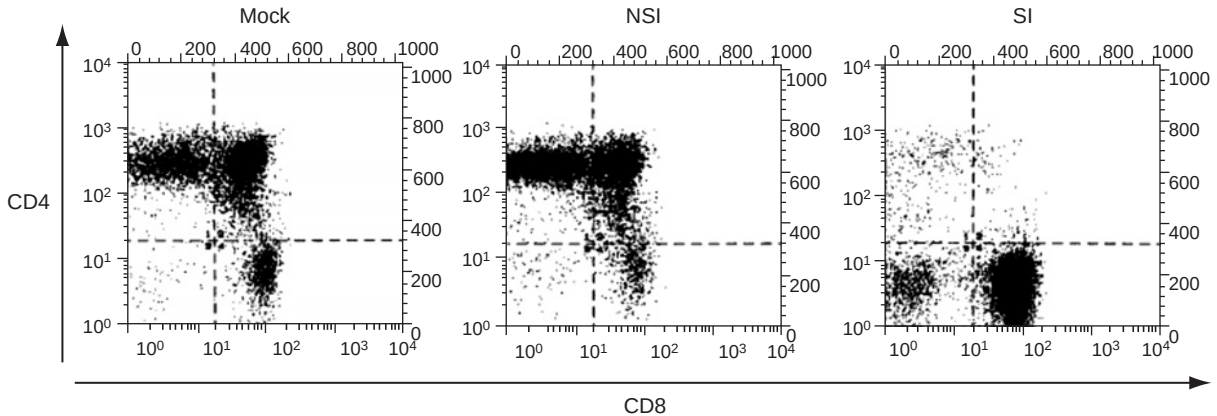


FIGURE 1 Two color flow cytometric analysis of CD4 PE and CD8 FITC expression on human thymocytes obtained from engraftment of SCID-hu mice. Six weeks after infection with mock, sequential nonsyncytium-inducing (NSI) and syncytium-inducing (SI) virus shows depletion of $CD4^+$ thymocytes with the cytopathic SI HIV-1. Adapted from Camerini *et al.* (2000).

may be responsible for its greater CPE since an increase in coreceptor affinity can increase apoptosis as well as viral replication (Holm *et al.*, 2004).

Disease progression following HIV-1 infection evolves from the asymptomatic period after a variable period. As progression occurs, the viral burden and CPE increase compared to the period of asymptomatic HIV-1 infection. Primary isolates of HIV-1 derived during early stages of infection utilize CCR5 to enter cells. CCR5 is part of a family of CC or β-chemokine receptors that belong to the much larger family of trimeric GTP-binding protein–coupled receptors (Samson *et al.*, 1996). CCR5 binds three chemokine ligands, CCL3, CCL4, and CCL5, formerly known as MIP-1α, MIP-1β, and RANTES. These molecules play important roles in the physiology of acute and chronic inflammatory process. As HIV-1 infection progresses, the virus switches from R5 to R5X4 or X4 phenotype in $\sim$50% of infected individuals (Connor *et al.*, 1997). Once R5X4 or X4 phenotype has evolved, $CD4^+$ T-cell depletion accelerates probably because CXCR4 is present on a larger subset of T cells, although the mechanisms of $CD4^+$ T-cell depletion are complex. Similarly, during HIV-1 infection of SCID-hu Thy/Liv grafts, both direct and indirect killing of thymocytes occur (Bonyhadi *et al.*, 1993; Jamieson *et al.*, 1997; Su *et al.*, 1995). During the rapid phase of $CD4^+$ thymocyte depletion, there is also a peak of viral replication, suggesting direct killing of HIV-1-infected thymocytes (Jamieson *et al.*, 1997). Moreover, the integrity of the thymic microenvironment is essential for T-cell development; perturbation of this environment by HIV-1 infection of the thymus can cause profound effects on T-cell development and function (Stanley *et al.*, 1993). HIV-1 infection of Thy/Liv organ causes depletion of hematopoietic progenitor cells (Jenkins *et al.*, 1998; Koka *et al.*, 1998; Stanley *et al.*, 1993) and destruction of thymic epithelial cells, thereby destroying this essential microenvironment (Joshi and Oleske, 1985; Stanley *et al.*, 1993). In association with HIV-1 infection, there is also an increase in MHC-I expression on the surface of thymic epithelial cells (Keir *et al.*, 2002a) and immature thymocytes (Keir *et al.*, 2002b; Kovalev *et al.*, 1999). The elevation of MHC-I levels may interfere with positive and negative selection of thymocytes causing a disruption of T-cell development or generation of dysfunctional T cells (Keir *et al.*, 2002a). Similarly, when MHC-I molecules are overexpressed on thymocytes in Tg mice, there is a concordant depletion of thymocytes (Schulz and Mellor, 1996).

The SCID-hu Thy/Liv model is also useful in the characterization of molecular determinants of HIV-1 required for replication *in vivo*. When a laboratory worker was accidentally infected with an attenuated infectious HIV-1 molecular clone, HXB2, the virus isolated from the worker displayed different cell tropism *in vitro* than HXB2. Moreover, HXB2, which has three defective genes, failed to replicate well in SCID-hu Thy/Liv mice (Su *et al.*, 1997). To map the determinants required for replication *in vivo*, recombinant viruses were made using the HXB2 genome with subgenomic fragments derived

from virus isolated from the laboratory worker (HXB2/LW). HXB2/LW clones containing the *env* gene derived from the laboratory worker replicated efficiently in SCID-hu mice. The enhanced replication was attributed to six unique mutations in the third variable region of gp120 (V3 loop) of the recombinant HXB2/LW.

Another study confirmed the importance of the *env* gene for replication of R5 HIV-1 in thymocytes derived from SCID-hu Thy/Liv mice *in vitro* and *in vivo* (Olivieri *et al*., 2007). Upon initial binding of gp120 to CD4, the complex undergoes a conformational change allowing gp120 to bind a coreceptor, usually CCR5 or CXCR4 (Dimitrov *et al*., 2001; Doranz *et al*., 1999; Kwong *et al*., 1998; Trkola *et al*., 1996). Further conformational changes in gp120 and in the viral transmembrane protein, gp41, mediate fusion of the viral lipid bilayer with the cytoplasmic membrane. Pre-AIDS R5 clones and AIDS-associated R5 clones differ in their *env* genes and as a result at least some AIDS-associated R5 clones exhibit greater efficiency and more rapid kinetics of the process of entry into cells (Olivieri *et al*., 2007). In a single round infection of SCID-hu-derived thymocytes *in vitro*, the AIDS-associated R5 clone, ACH142-*E11, exhibited significantly greater infection efficiency than two pre-AIDS clones derived from the same patient, 32D2 and 8G9. To characterize the phenotype, recombinant viruses bearing the V1-V5 *env* encoding segments of pre-AIDS- and AIDS-associated patient derived R5 HIV-1 clones were inserted into the JR-CSF R5 HIV-1 molecular clone and used to infect SCID-hu Thy/Liv mice (Olivieri *et al*., 2007). The late stage, *E11 V1-V5 recombinant virus was more cytopathic for $CD4^+$ thymocytes and replicated to higher levels in SCID-hu Thy/Liv mice than the pre-AIDS 32D2 recombinant virus or the parental R5 HIV-1 molecular, JR-CSF (Fig. 2).

IV. The hu-PBL-SCID Mouse

The hu-PBL-SCID mouse is generated by intraperitoneal injection of human PBL into SCID mice (Mosier *et al*., 1988). During the three weeks after intraperitoneal injection, the majority of PBL reside in the peritoneal cavity. After one month, human cells begin to appear in the liver and spleen in some cases (Tary-Lehmann and Saxon, 1992). The majority of new emigrants into the liver and spleen are single positive, $CD4^+$ or $CD8^-$ and express HLA-DR and CD45RO (Duchosal *et al*., 1992; Hoffmann-Fezer *et al*., 1993; Tary-Lehmann and Saxon, 1992). Furthermore, there is an expansion of $CD3^+$ T cells, but smaller numbers of human monocytes, NK cells, and B cells that secrete human immunoglobulin and generate secondary antibody responses persist (Mosier *et al*., 1988, 1991; Reinhardt *et al*., 1994). The engrafted cells not only populate the peritoneal cavity but also reside within the lymph nodes, spleen, and bone marrow

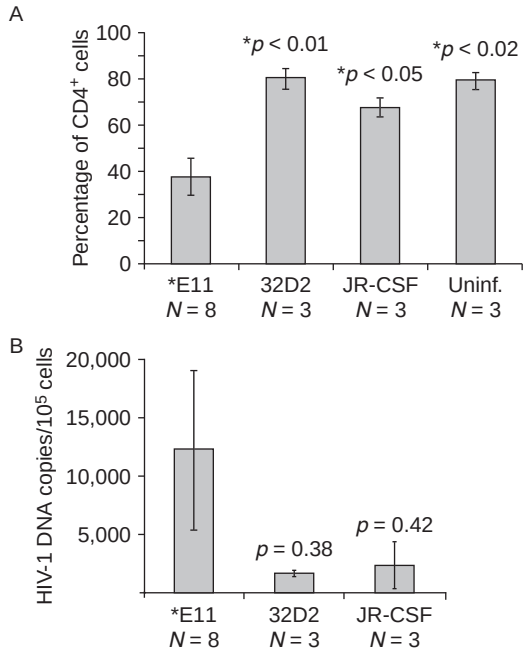


FIGURE 2 JR-CSF (*E11 V1-V5) achieved higher replication and induced greater $CD4^+$ thymocyte loss in SCID-hu Thy/Liv graft infections than JR-CSF (32D2 V1-V5) or JR-CSF. At eight weeks postinfection, a piece of each infected SCID-hu Thy/Liv graft was removed and a single cell suspension was made. This was analyzed for cell surface expression of CD4 by flow cytometry and for the presence of HIV-1 strong-stop DNA. (A) Summary of percentage of $CD4^+$ cells in all infected grafts at eight weeks postinfection. (B) HIV-1 strong-stop DNA detected per 10^5 cells in each infected graft at eight weeks postinfection. Cells from the same single cell suspension used in (A) were used to make a total intracellular DNA preparation which was measured by a real-time PCR assay to detect HIV-1 strong-stop DNA and β-globin copies. The number of HIV-1 DNA copies was normalized to cell number by measuring the number of β-globin genes in the DNA sample. These values are shown minus the background level of detection as determined in uninfected samples. Asterisks indicate values that were significantly different by Student's *t*-test from the uninfected sample (*p* values are as indicated). The *N* values below each bar are equal to the number of samples in each set. Adapted from Olivieri *et al.* (2007).

(Mosier, 1996; Rizza *et al.*, 1996). Autologous skin transplants have been used with hu-PBL-SCID mice (Delhem *et al.*, 1998), as they have with SCID-hu mice (Carballido *et al.*, 2003, 2000), to augment the number of human antigen presenting cells and increase the complexity of the engrafted human immune system. The hu-PBL-SCID model with various modifications has been used extensively to study HIV-1 pathogenesis, anti-HIV therapy, immune responses, and vaccine efficacy *in vivo* (Boyle *et al.*, 1995; Delhem *et al.*, 1998; Gauduin *et al.*, 1997; Koup *et al.*, 1996; Lapenta *et al.*, 1999,

2003; Mosier, 1996; Mosier *et al*., 1991, 1993a,b; Okamoto *et al*., 1998; Parren *et al*., 1995; Picchio *et al*., 2000; Poignard *et al*., 1999; Reinhardt *et al*., 1994; Rizza *et al*., 1996; Safrit *et al*., 1993; Santini *et al*., 2000; Vieillard *et al*., 1999; Yoshida *et al*., 2003, 2005).

A distinguishing advantage of the hu-PBL-SCID mice model is the ability to evaluate anti-HIV-1 effector immune functions that have been primed by vaccination or created *in vitro*. Adoptive transfer of cells or antibodies before or after HIV-1 infection of hu-PBL-SCID mice has been used as a test of antiviral immune responses and immune mediators. For example, four HIV-negative volunteers were immunized with vaccinia virus expressing HIV-1 gp160 and boosted with recombinant gp160. PBL from the immunized donors were used to create hu-PBL-SCID mice (Mosier *et al*., 1993b). Mice that received PBL from three of the four human donors were protected from infection when challenged with HIV-1 shortly after the second immunization. Passive immunization with IgG1-b12, a recombinant human monoclonal antibody directed to an epitope overlapping the CD4 binding site, protected hu-PBL-SCID mice from challenge with HIV-1 (Gauduin *et al*., 1997; Parren *et al*., 1995). Similar results were demonstrated with a monoclonal antibody directed to the V3 loop of gp120 (Gauduin *et al*., 1995; Safrit *et al*., 1993). Anti-HIV-1 cytotoxic T lymphocyte (CTL) has also been transferred to hu-PBL-SCID mice and was protective against subsequent challenge with HIV-1 (McKinney *et al*., 1999, Van Kuyk *et al*., 1994). The protection was found to be short lived, however, and partially nonspecific. Vaccine efficacy trials based on HIV-pulsed dendritic cells (DC) have also been studied in hu-PBL-SCID mice (Lapenta *et al*., 2003; Yoshida *et al*., 2003). When HIV-1 negative human peripheral blood mononuclear cells (PBMC) were transplanted into SCID mice spleens with inactivated HIV-1 pulsed autologous DC, the combination provided protective immunity against R5 HIV-1 (Yoshida *et al*., 2003).

V. NOD/LtSz-SCID Mice

The levels of engraftment of circulating human cells in SCID mice have been shown to be limited by innate immunity, including NK cells, macrophages, and complement (Christianson *et al*., 1996; Shultz *et al*., 1995). To improve the efficiency of engraftment, Shultz and colleagues at Jackson Labs crossed SCID mice with a variety of mouse strains and found that NOD-SCID mice allowed higher levels of human lymphoid cell engraftment. The NOD/LtSz-SCID/SCID mice they created exhibited five- to tenfold greater stable levels of engrafted human spleen cells or PBMC as well as increased migration of these cells to mouse lymphoid organs and higher levels of human immunoglobulins (Greiner *et al*., 1995; Hesselton *et al*., 1995; Shultz *et al*., 1995). $CD4^+$ T cells, however, were not maintained at a

normal ratio to $CD8^+$ T cells, which was detrimental to studies of HIV-1. To enhance $CD4^+$ T-cell engraftment, NOD-SCID β_2-microglobulin-null (NOD-SCID-$\beta 2m^{null}$) mice were subsequently created by the same group (Christianson *et al.*, 1997). NOD-SCID and NOD-SCID-$\beta 2m^{null}$ mice are significantly better than simple SCID mice for the creation of hu-PBL-SCID mice as a model for HIV-1 infection, but their use as recipients of Thy/Liv grafts to create SCID-hu (Thy/Liv) mice and in long-term experiments is limited by their short life span, averaging 37 weeks.

VI. Rag2$^{-/-}$ $\gamma C^{-/-}$ and NOD-SCID $\gamma C^{-/-}$ Mice

Two further improvements have been made in the immunodeficient mice used to create chimeric hu-PBL-SCID or SCID-hu mice. The first is the use of the recombinase activating gene (Rag)-1 or Rag-$2^{-/-}$ mutation instead of the SCID mutation. The original Rag knockout mice were not useful as hosts for human cells due to high levels of innate immunity, but subsequent creation of Balb/C Rag-$2^{-/-}$ and NOD-$Rag^{-/-}$ mice by several groups has eliminated this problem (Ito *et al.*, 2002; Shultz *et al.*, 2000; Traggiai *et al.*, 2004). The $Rag^{-/-}$ mutations are superior to the SCID mutation because they are not leaky like the SCID mutation, in which low levels of rearranged immunoglobulin and TCR genes accumulate over time. Furthermore, SCID mice are radiation sensitive since the defect is in an enzyme that is needed in DNA repair (DNA-activated protein kinase catalytic subunit), while Rag mice are not more radiation sensitive than wild type. The next significant advance was the use of knockout mutations in the common interleukin receptor (IL) gamma chain (γc), a component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-23, to further cripple the mouse immune system. The insertion of the $\gamma c^{-/-}$ mutation into Balb/C $Rag^{-/-}$ or NOD-SCID mice has now created long-lived, severely immunodeficient mice capable of supporting multilineage human hematopoiesis following intravenous, intraperitoneal, or intrahepatic injection of $CD34^+$ HSC into adult or newborn mice (Ishikawa *et al.*, 2005; Ito *et al.*, 2002; Shultz *et al.*, 2005; Traggiai *et al.*, 2004). Recipient $Rag^{-/-}$ $\gamma c^{-/-}$ or NOD-SCID $\gamma c^{-/-}$ mice were reproducibly engrafted with human myeloid cells, DC, red blood cells, platelets, B cells, and T cells. Moreover, recipient mice exhibited formation of primary and secondary lymphoid organs, adaptive immune responses, and persistence of self-renewing HSC. Three groups have now infected $Rag^{-/-}$ $\gamma c^{-/-}$ or NOD-SCID $\gamma c^{-/-}$ mice with both R5 and X4 HIV-1 (Baenziger *et al.*, 2006; Berges *et al.*, 2006; Watanabe *et al.*, 2007). They observed sustained HIV-1 infection, $CD4^+$ T-cell depletion in peripheral blood, spleen, and thymus, and the appearance of anti-HIV-1 antibodies. This new $Rag^{-/-}$ $\gamma c^{-/-}$ or NOD-SCID $\gamma c^{-/-}$ mouse model of HIV-1 infection is a very promising development.

VII. Humanized Immune Competent Mice and Rats

An alternative approach to creating a mouse model of HIV-1 infection is to use immune competent mice Tg for key human genes required for HIV-1 replication. Initial attempts using human CD4 expressed by mouse cells *in vitro* or in Tg mice, however, showed that CD4 alone was not sufficient to allow replication of HIV-1 in mouse cells (Lores *et al*., 1992; Maddon *et al*., 1986). It has subsequently been shown that HIV-1 replication in mouse cells is hindered at multiple steps including viral entry, transcription, RNA processing, and assembly (Bieniasz and Cullen, 2000). The entry deficiency could be remedied by the addition of human CCR5 since mouse CCR5 does not bind HIV-1 but mouse CXCR4 is functional for HIV-1 entry (Atchison *et al*., 1996; Bieniasz *et al*., 1997). One group reported that human CD4/CCR5 double Tg mice supported R5 HIV-1 replication but the levels of virus produced were very low (Browning *et al*., 1997). Another block within murine cells is in transcription of the proviral genome since mouse cyclin T1 does not interact effectively with HIV-1 *Tat* and TAR (Garber *et al*., 1998). Mouse NIH 3T3 fibroblasts expressing human CD4, CCR5, and cyclin T1 were able to support reverse transcription, integration, proviral transcription, and translation but were not able to assemble and release virions efficiently (Mariani *et al*., 2000). These murine cells expressed *Gag* and *Gag-Pol*, but they accumulated in the cells, perhaps because they were inefficiently processed by viral protease (Bieniasz and Cullen, 2000; Moosmayer *et al*., 1991). Mouse–human heterokaryon formation is able to overcome the viral assembly block, suggesting that mouse cells lack a factor needed for efficient assembly or release of HIV-1 (Bieniasz and Cullen, 2000; Mariani *et al*., 2001). Recently, a human splicing inhibitor, p32, was shown to increase the level of unspliced viral transcripts in mouse cells suggesting that another block to HIV-1 replication in mice is over efficient splicing (Zheng *et al*., 2003). The unspliced HIV-1 RNA encodes the *gag* and *pol* genes and is also the viral genome so its level is critical for the production of infectious HIV-1. Despite all these advances in understanding the defects in mouse cells, there is currently no efficient immune competent mouse model of HIV-1 replication.

Another model for HIV-1 infection is the human CD4/CCR5 Tg rat, *Rattus norvegicus*. The rat fibroblast cell line, Rat2, and neuroblastoma cell line, B50, can produce low levels of infectious HIV-1 (Bieniasz and Cullen, 2000; Mariani *et al*., 2000; Mizrachi *et al*., 1992). Rat cells exhibit the same blocks to HIV-1 replication as mouse cells but they are less severe. Coexpression of human CD4 and CCR5 allowed low-level HIV-1 replication in rat cells *in vitro* and the addition of human cylin-T1 was also beneficial (Keppler *et al*., 2001). Coexpression of human CD4 and CCR5 in Tg rats leads to expression on rat $CD4^+$ T cells, macrophages, and microglia but not on rat $CD8^+$ T cells or B cells. Moreover, *ex vivo* infection of primary macrophages

or microglia but not T cells from hu-CD4/CCR5 Tg rat resulted in productive infection and generation of replication competent virus (Keppler *et al.*, 2002, 2005). Nevertheless, infection of human CD4/CCR5 Tg rats *in vivo* is transient and low levels of virus are produced.

VIII. HIV-1 Tg Mice

Several HIV-1 Tg mouse models have been developed to evaluate HIV-induced pathogenesis in an *in vivo* setting. As noted in the previous section, attempts to develop a humanized immune competent mouse model of HIV-1 infection were limited by the absence of functional murine homologues of the HIV-1 receptor, coreceptor, host cell factors required for Tat-mediated transcriptional activation, splicing silencer, and other factors that are still unknown. To bypass these constraints, Tg mice were constructed in 1988 with a T lymphoblastoid cell line adapted infectious X4 HIV-1 molecular clone called NL4–3 (Leonard *et al.*, 1988). All 7 founder mice showed no phenotype but nearly half of the F1 offspring of one founder developed multiorgan disease and died by day 25 of life. These mice exhibited epidermal hyperplasia, lymphadenopathy, splenomegaly, pneumonitis, and growth retardation. Infectious HIV-1 was recovered from the skin, spleen, and lymph nodes of affected mice. A noninfectious version of the NL4–3 HIV-1 clone with a deletion in the *gag* and *pol* genes was also used to make Tg mice (Santoro *et al.*, 1994). Mice homozygous for this Tg exhibited a phenotype very similar to the F1 offspring of the infectious NL4–3 Tg mice with notable skin and lymphoid organ involvement. It was later found that treatment of these NL4–3 Δ*gag-pol* Tg mice with human chorionic gonadotropin (hCG) reduced HIV-1 RNA and protein expression, prevented death, and reversed other symptoms, including growth retardation and skin lesions (De *et al.*, 1997). Further studies revealed that tumor necrosis factor-α (TNF-α) was an important mediator of disease in NL4–3 Δ*gag-pol* Tg mice and that hCG reversed the symptoms by lowering TNF-α levels (De *et al.*, 2002). Moreover, a similar decrease in HIV-1 gene expression and amelioration of symptoms resulted from administration of anti-TNF-α antibody.

A different Tg mouse was created with the NL4–3 genome without its LTRs under the regulatory control of the human CD4 promoter and mouse CD4 enhancer (Hanna *et al.*, 1998a). This mouse, designated CD4C/HIVwt, developed severe AIDS-like disease, including loss of $CD4^+$ T cells, growth retardation, diarrhea, pneumonitis, nephritis, and early death. Numerous studies have shown that the primary determinant of disease in these animals is the *nef* gene, that its action is dependent on the PXXP SH3 binding domain of Nef and is mediated in part by T-cell activation (Hanna *et al.*, 1998b, 2001, 2004; Weng *et al.*, 2004). Another Tg mouse line was created

from the X4 HIV-1 strain SF-2, bearing deletions in all three structural genes, *gag*, *pol*, and *env* (Tinkle *et al.*, 1997). These mice also exhibited depletion of mature T cells, which died by apoptosis.

More recently, the R5 HIV-1, clone JR-CSF, was used to create a line of Tg mice (Paul *et al.*, 2000). In contrast to the other HIV-1 Tg mice described above, however, these mice did not exhibit any disease, although they had high levels of plasma viremia, which could be increased further by stimulation with *Staphylococcus* enterotoxin B or infection with *Mycobacterium tuberculosis*. The JR-CSF Tg mice produced infectious HIV-1 from T cells and macrophages and production of virus by macrophages and brain microglial cells *in vitro* could be stimulated by treatment with LPS or GM-CSF (Osiecki *et al.*, 2005; Wang *et al.*, 2003). The JR-CSF Tg mouse was crossed with a Tg mouse that expresses human cyclin-T1 under the control of the mouse CD4 enhancer and promoter (Sun *et al.*, 2006). These doubly Tg mice displayed increased HIV-1 expression in $CD4^+$ cells compared to mice expressing JR-CSF alone and also exhibited depletion of $CD4^+$ T cells.

Several groups created Tg mice expressing a single HIV-1 gene, notably *nef*, *tat*, *env* (gp120 encoding portion), and *vpr* (Keswani *et al.*, 2006; Kim *et al.*, 2003; Lindemann *et al.*, 1994; Skowronski *et al.*, 1993; Toggas *et al.*, 1994; Weng *et al.*, 2004; Yasuda *et al.*, 2001). These mice have been useful in modeling particular aspects of HIV-1-mediated disease. The HIV-1 *nef* gene has been implicated in contributing to AIDS-like disease in Tg mice by many studies (Hanna *et al.*, 1998b, 2001, 2004; Weng *et al.*, 2004). A fatal AIDS-like disease occurred in Tg mice harboring a functional *nef* (CD4C/HIV^{nef}), but not in HIV-1 Tg mice that lacked functional Weng *et al.*, 2004). Peripheral $CD4^+$ T cells isolated from CD4C/HIV^{nef} Tg mice exhibit an activated/memory phenotype with higher expression of CD25 and CD69 on $CD4^+$ T cells in the absence of TCR-mediated stimulation. Thymocytes from these Tg mice also displayed hypersensitivity to stimulation with CD3 MAb manifest by a constitutive increase in tyrosine phosphorylation of several substrates including LAT and p44/p42 MAPK (Erk-1/Erk-2). The chronic activation of Tg $CD4^+$ T cells and their hypersensitivity to TCR/CD3-mediated signals caused apoptosis after few cell divisions. The importance of *nef* is clear since mutation in the SH3 ligand-binding domain of *nef* results in abrogation of hypersensitivity to CD3 stimulation and failure to develop disease (Hanna *et al.*, 2001).

Among the many HIV tat Tg mice, those that express *tat* under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter in a doxycycline (Dox)-dependent manner are particularly interesting (Kim *et al.*, 2003). These mice were developed to study neuropathic disorders associated with HIV-1 infection. The expression of *tat* occurred exclusively in astrocytes and was Dox dependent. Expression of *tat* in the brain caused damage to the cerebellum, cortex, astrocytes, degeneration of neuronal dendrites, neuronal apoptosis, and increased infiltration of activated

monocytes and T lymphocytes similar to what is seen in the brains of AIDS patients. HIV tat Tg mice, therefore, support the hypothesized role of Tat protein in HIV-1 neuropathogenesis and may be helpful in delineating the molecular mechanisms of Tat-induced neurotoxicity and apoptosis.

An HIV vpr Tg mouse was developed to investigate CPE mediated by *vpr in vivo* (Yasuda *et al.*, 2001). X4-HIV-1, clone NL4–3 *vpr*, was under control of the mouse CD4 enhancer/promoter in the Tg mice. HIV vpr Tg mice had reduced numbers of T cells both in the thymus and in the peripheral blood due to increased apoptosis. The highest expression of *vpr* was observed in the thymus followed by the lymph node (50-fold lower) and the spleen (100-fold lower). Crossing HIV vpr Tg mice with *gld*-mice, which have a deficient FasL gene, caused thymic atrophy suggesting that Fas–FasL interactions are not required for apoptosis induced by *vpr*. Increased expression of Bcl-x, Bax, and caspase-1 genes, however, was observed in thymii of these Tg mice, suggesting the involvement of mitochondrial pathways in *vpr*-mediated apoptosis.

In a study similar to the *tat* Tg mouse described above, the effects of HIV-1 gp120 in the brain were assessed with GFAP promoter-driven HIV-1 *env* (gp120 encoding portion) mice (Toggas *et al.*, 1994). These mice expressed gp120 in astrocytes and exhibited reactive astrocytosis, microglial cell hypertrophy, and neuronal damage similar to that seen in AIDS patients. More recently, these mice have been used as a model of HIV-associated sensory neuropathy, the most common neurological complication of HIV-1 infection following treatment with didanosine (DDI) or other dideoxynucleoside RT inhibitors (Keswani *et al.*, 2006). Administration of DDI caused GFAP-gp120 Tg mice to have decreased intraepidermal nerve fiber density and distal degeneration of unmyelinated sensory axons similar to the findings in patients.

IX. Rabbit Model of HIV Pathogenesis

An alternative small animal model that has been utilized for the study of HIV is the rabbit model. Similar to the rodent model, viral entry is the main block to successful infection and has been overcome by having rabbit cells express both human CD4 CCR5 (Speck *et al.*, 1998). These rabbit cells exhibited a low level of viral replication similar to what was previously seen in a variety of human $CD4^+$ rabbit cells *in vitro* (Hague *et al.*, 1992; Kulaga *et al.*, 1988; Yamamura *et al.*, 1991). Nevertheless, human CD4 Tg rabbits were created but they do not support sustained or high-level replication of HIV-1 (Dunn *et al.*, 1995; Leno *et al.*, 1995; Snyder *et al.*, 1995). Since rabbits have a short gestational period, they have been utilized to study maternal-to-child transmission (MTCT) of HIV-1 (Simpson *et al.*, 1997). Vertical transmission of HIV-1 from infected human T cells *in utero* was

demonstrated. Upon *in utero* infection, rabbit offspring had pathological changes varying from subacute interstitial pneumonias to lymphoid hyperplasia/depletion and thymic atrophy.

X. Conclusion

In the endeavor to attain a small animal model to mimic HIV and AIDS in humans, the creation of genetically modified and chimeric small laboratory animals has been a fruitful approach. The immunodeficient mouse models offer the ability to engraft hematopoietic cells along with thymocytes or PBL to study specific aspects of HIV-1 infection. The recent creation of NOD-SCID $\gamma c^{-/-}$ and $Rag2^{-/-}$ $\gamma c^{-/-}$ mice allows higher efficiency of engraftment of human cells and holds promise for studies of HIV-1 replication and pathogenesis. Tg mice have also been useful for the study of specific aspects of HIV-1 disease. Other models, including Tg rabbits and rats, may become useful in the future. It is clear that small animal models will continue to play an important role in the study of HIV-1 replication and pathogenesis.

References

Aldrovandi, G. M., and Zack, J. A. (1996). Replication and pathogenicity of human immunodeficiency virus type 1 accessory gene mutants in SCID-hu mice. *J. Virol.* **70**(3), 1505–1511.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berge, E. A. (1996). CC CKR5: A RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.

Alkhatib, G., Berger, E. A., Murphy, P. M., and Pease, J. E. (1997). Determinants of HIV-1 coreceptor function on CC chemokine receptor 3. Importance of both extracellular and transmembrane/cytplasmic regions. *J. Biol. Chem.* **272**, 20420–20426.

Atchison, R. E., Gosling, J., Monteclaro, F. S., Franci, C., Digilio, L., Charo, I. F., and Goldsmith, M. A. (1996). Multiple extracellular elements of CCR5 and HIV-1 entry: Dissociation from response to chemokines. *Science* **274**, 1924–1926.

Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M. O., Behnke, S., Frey, J., Oxenius, A., Joller, H., Aguzzi, A., Manz, M. G., and Speck, R. F. (2006). Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted $Rag2^{-/-}\gamma c^{-/-}$ mice. *Proc. Natl. Acad. Sci. USA* **103**, 15951–15956.

Bazan, H. A., Alkhatib, G., Broder, C. C., and Berger, E. A. (1998). Patterns of CCR5, CXCR4, and CCR3 usage by envelope glycoproteins from human immunodeficiency virus type 1 primary isolates. *J. Virol.* **72**, 4485–4491.

Berges, R. K., Wheat, W. H., Palmer, B. E., Connick, E., and Akkina, R. (2006). HIV-1 infection and CD4 T cell depletion in the humanized $Rag2^{-/-}\gamma c^{-/-}$ (RAG-hu) mouse model. *Retrovirology* **3**, 76.

Berkowitz, R. D., Beckerman, K. P., Schall, T. J., and McCune, J. M. (1998). CXCR4 and CCR5 expression delineates targets for HIV-1 disruption of T cell differentiation. *J. Immunol.* **161**(7), 3702–3710.

Bieniasz, P. D., and Cullen, B. R. (2000). Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. *J. Virol.* **74**, 9868–9877.

Bieniasz, P. D., Fridell, R. A., Anthony, K., and Cullen, B. R. (1997). Murine CXCR-4 is a functional coreceptor for T-cell-tropic and dual-tropic strains of human immunodeficiency virus type 1. *J. Virol.* **71**, 7097–7100.

Bonyhadi, M. L., Rabin, L., Salimi, S., Brown, D. A., Kosek, J., McCune, J. M., and Kaneshima, H. (1993). HIV induces thymus depletion *in vivo*. *Nature* **363**, 728–732.

Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature* **301**, 527–530.

Boyle, M. J., Connors, M., Flanigan, M. E., Geiger, S. P., Ford, H., Jr., Baseler, M., Adelsberger, J., Davey, R. T., Jr., and Lane, H. C. (1995). The human HIV/peripheral blood lymphocyte (PBL)-SCID mouse. A modified human PBL-SCID model for the study of HIV pathogenesis and therapy. *J. Immunol.* **154**, 6612–6623.

Browning, J., Horner, J. W., Pettoello-Mantovani, M., Raker, C., Yurasov, S., DePinho, R. A., and Goldstein, H. (1997). Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl. Acad. Sci. USA* **94**, 14637–14641.

Camerini, D., Su, H.-P., Gamez-Torre, G., Johnson, M. L., Zack, J. A., and Chen, I. S. (2000). Human immunodeficiency virus Type 1 pathogenesis in SCID-hu mice correlates with syncytium-inducing phenotype and viral replication. *J. Virol.* **74**(7), 3196–3204.

Carballido, J. M., Namikawa, R., Carballido-Perrig, N., Antonenko, S., Roncarolo, M. G., and de Vries, J. E. (2000). Generation of primary antigen-specific human T- and B-cell responses in immunocompetent SCID-hu mice. *Nat. Med.* **6**, 103–106.

Carballido, J. M., Biedermann, T., Schwarzler, C., and De Vries, J. E. (2003). The SCID-hu skin mouse as a model to investigate selective chemokine mediated homing of human T-lymphocytes to the skin *in vivo*. *J. Immunol. Methods* **273**, 125–135.

Choe, H., Farzan, Y., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., *et al.* (1996). The beta-chemokne receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135–1148.

Choe, H., Farzan, M., Konkel, M., Martin, K., Sun, Y., Marcon, M., Cayabyab, M., Berman, M., Dorf, N., Gerard, C., Gerard, N., and Sodroski, J. (1998). The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dual-tropic human immunodeficiency virus type 1. *J. Virol.* **72**, 6113–6118.

Christianson, S. W., Greiner, D. L., Schweitzer, I. B., Gott, B., Beamer, G. L., Schweitzer, P. A., Hesselton, R., and Shultz, L. D. (1996). Role of natural killer cells on engraftment of human lymphoid cells and on metastasis of human T-lymphoblastoid leukemia cells in C57BL/6J-scidMice and in C57BL/6J-scid bgMice. *Cell. Immunol.* **171**, 186–199.

Christianson, S. W., Greiner, D. L., Hesselton, R. M., Leif, J. H., Wagar, E. J., Schweitzer, I. B., Rajan, T. V., Gott, B., Roopenian, D. C., and Shultz, L. D. (1997). Enhanced human $CD4^+$ T cell engraftment in β_2-microglobulin-deficient NOD-*scid* mice. *J. Immunol.* **158**, 3578–3586.

Connor, R. I., Mohri, H., Cao, Y., and Ho, D. (1993). Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. *J. Virol.* **67**, 1772–1777.

Connor, R. I., Sheridan, D., Ceradinid, D., Choe, S., and Landau, N. R. (1997). Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *J. Exp. Med.* **185**, 621–628.

Custer, R. P., Bosma, G. C., and Bosma, M. J. (1985). Severe combined immunodeficiency (SCID) in the mouse. Pathology, reconstitution, neoplasms. *Am. J. Pathol.* **120**, 464–477.

De, S. K., Wohlenberg, C. R., Marinos, N. J., Doodnauth, D., Bryant, J. L., and Notkins, A. L. (1997). Human chorionic gonadotropin hormone prevents wasting syndrome and death in HIV-1 transgenic mice. *J. Clin. Invest.* **99**, 1484–1491.

De, S. K., Devadas, K., and Notkins, A. L. (2002). Elevated levels of tumor necrosis factor alpha (TNF-α) in human immunodeficiency virus type 1-transgenic mice: Prevention of death by antibody to TNF-α. *J. Virol.* **76**, 11710–11714.

Delhem, N., Hadida, F., Gorochov, G., Carpentier, F., de Cavel, J. P., Andreani, J. F., Autran, B., and Cesbron, J. Y. (1998). Primary Th1 cell immunization against HIV gp160 in SCID-hu mice coengrafted with peripheral blood lymphocytes and skin. *J. Immunol.* **161**, 2060–2069.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Dimarzio, P., Marmom, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., *et al.* (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661–666.

Dimitrov, A. S., Xiao, X., Dimitrov, D. S., and Blumenthal, R. (2001). Early intermediates in HIV-1 envelope glycoprotein-mediated fusion triggered by CD4 and co-receptor complexes. *J. Biol. Chem.* **276**, 30335–30341.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. C., and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149–1158.

Doranz, B. J., Baik, S. S., and Dorms, R. W. (1999). Use of a gp120 binding assay to dissect the requirements and kinetics of human immunodeficiency virus fusion events. *J. Virol.* **73**, 10346–10358.

Dorshkind, K., Keller, G. M., Phillips, R. A., Miller, R. G., Bosma, G. C., O'Toole, M., and Bosma, M. J. (1984). Functional status of cells from lymphoid and myeloid tissues in mice with severe combined immunodeficiency disease. *J. Immunol.* **132**, 1804–1808.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR5. *Nature* **381**, 667–673.

Duchosal, M. A., Eming, S. A., McConahey, P. J., and Dixon, F. J. (1992). Characterization of hu-PBL-SCID mice with high human immunoglobulin serum levels and graft-versus-host disease. *Am. J. Pathol.* **141**, 1097–1113.

Dunn, C. S., Mehtali, M., Houdebine, L. M., Gut, J. P., Kirn, A., and Aubertin, A. M. (1995). Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits. *J. Gen. Virol.* **76**, 1327–1336.

Edinger, A. L., Hoffman, T. L., Sharron, M., Lee, B., Yi, Y., Choe, W., Kolson, D. L., Mitrovic, B., Zhou, Y., Faulds, D., Collman, R. G., Hesselgesser, J., *et al.* (1998). An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol.* **72**, 7934–7940.

Farzan, M., Choe, H., Martin, K., Marcou, L., Hoffmann, W., Karlsson, G., Sun, Y., Barrett, P., Marchand, N., Sullivan, N., Gerard, N., Gerard, C., *et al.* (1997). Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J. Exp. Med.* **186**, 405–411.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.

Frade, J. M. R., Llorente, M., Mellado, M., Alcami, J., Gutierrez-Ramos, J. C., Zaballos, A., Real, G., and Martinez, A. C. (1997). The amino-terminal domain of the CCR2 chemokine receptor acts as coreceptor for HIV-1 infection. *J. Clin. Invest.* **100**, 497–502.

Garber, M. E., Wei, P., KewlRamani, V. N., Mayall, T. P., Herrmann, C. H., Rice, A. P., Littman, D. R., and Jones, K. A. (1998). The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev.* **12**, 3512–3527.

Gauduin, M. C., Safrit, J. T., Wei, R., Fung, M. S., and Koup, R. A. (1995). Pre- and postexposure protection against human immunodeficiency virus type 1 infection mediated by a monoclonal antibody. *J. Infect. Dis.* **171**, 1203–1209.

Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R., and Koup, R. A. (1997). Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. *Nat. Med.* **3**, 1389–1393.

Greiner, D. L., Shultz, L. D., Yates, J., Appel, M. C., Perdrizet, G., Hesselton, R. M., Schweitzer, I., Beamer, W. G., Shultz, K. L., and Pelsue, S. C. (1995). Improved engraftment of human spleen cells in Nod/LtSz-scid/scid mice as compared with C.B-17-scid/scid mice. *Am. J. Pathol.* **146**, 888–902.

Hague, B. F., Swasdikosol, S., Brown, T. J., Lee, D., Recker, D. P., and Kindt, T. J. (1992). CD4 and its role in infection of rabbit cell lines by human immunodeficiency virus type 1. *Immunology* **89**, 7963–7967.

Hanna, Z., Kay, D. G., Cool, M., Jothy, S., Rebai, N., and Jolicoeur, P. (1998a). Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease. *J. Virol.* **72**, 121–132.

Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S., and Jolicoeur, P. (1998b). *Nef* harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* **95**, 163–175.

Hanna, Z., Weng, X., Kay, D. G., Poudrier, J., Lowell, C., and Jolicoeur, P. (2001). The pathogenicity of human immunodeficiency virus (HIV) type 1 *Nef* in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. *J. Virol.* **75**, 9378–9392.

Hanna, Z., Priceputu, E., Kay, D. G., Poudrier, J., Chrobak, P., and Jolicoeur, P. (2004). *In vivo* mutational analysis of the N-terminal region of HIV-1 *Nef* reveals critical motifs for the development of AIDS-like disease in CD4C/HIV transgenic mice. *Virology* **327**, 273–286.

He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J., *et al.* (1997). CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385**, 645–649.

Hesselton, R. M., Greiner, D. L., Mordes, J. P., Rajan, T. V., Sullivan, J. L., and Shultz, L. D. (1995). High levels of human peripheral blood mononuclear cell engraftment and enhanced susceptibility to human immunodeficiency virus type 1 infection in NOD/LtSz-scid/scid mice. *J. Infect. Dis.* **172**, 974–982.

Hoffmann-Fezer, G., Gall, C., Zengerle, U., Kranz, B., and Thierfelder, S. (1993). Immunohistology and immunocytology of human T cell chimerism and graft-versus-host disease. *Blood* **81**, 3440–3448.

Holm, G. H., Zhang, C., Gorry, P. R., Peden, K., Schols, D., De Clercq, E., and Gabuzda, D. (2004). Apoptosis of bystander T cells induced by human immunodeficiency virus type 1 with increased envelope/receptor affinity and coreceptor binding site exposure. *J. Virol.* **78**, 4541–4551.

Horuk, R., Hesselgesser, R. J., Zhou, Y., Faulds, D., Halks-Miller, M., Harvey, S., Taub, D., Samson, M., Parmentier, M., Rucker, J., Doranz, B. J., and Doms, R. W. (1998). The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. *J. Biol. Chem.* **273**, 386–391.

Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L. D., and Harada, M. (2005). Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ-chain^{null} mice. *Blood* **106**, 1565–1573.

Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., Heike, T., and Nakahata, T. (2002). NOD/SCID/γc null mouse: An excellent recipient mouse model for engraftment of human cells. *Blood* **100**, 3175–3182.

Jamieson, B. D., Pang, S., Aldrovandi, G. M., Zha, J., and Zack, J. A. (1995). *In vivo* pathogenic properties of two clonal human immunodeficiency virus type 1 isolates. *J. Virol.* **69**, 6259–6264.

Jamieson, B. D., Uittenbogaart, C. H., Schmid, I., and Zack, J. A. (1997). High viral burden and $CD4^+$ cell depletion in human immunodeficiency virus type 1-infected SCID-hu mice suggest direct viral killing of thymocytes *in vivo*. *J. Virol.* **71**, 8245–8253.

Jenkins, M., Hanley, M. B., Moreno, M. B., Wieder, E., and McCune, J. M. (1998). Human immunodeficiency virus-1 infection interrupts thymopoiesis and multilineage hematopoiesis *in vivo*. *Blood* **91**, 2672–2678.

Joshi, V. V., and Oleske, J. M. (1985). Pathologic appraisal of the thymus gland in acquired immunodeficiency syndrome in children. A study of four cases and a review of the literature. *Arch. Pathol. Lab. Med.* **109**, 142–146.

Kaneshima, H., Su, L., Bonyhadi, L., Connor, R. I., Ho, D. D., and McCune, J. (1994). Rapid-high, syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathocitiy in the human thymus of the SCID-hu mouse. *J. Virol.* **68**, 8188–8192.

Keir, M. E., Rosenberg, M. G., Sandberg, J. K., Jordan, J. A., Wiznia, A., Nixon, D. F., Stoddart, C. A., and McCune, J. M. (2002a). Generation of $CD3^+$ $CD8^{low}$ thymocytes in the HIV type 1-infected thymus. *J. Immunol.* **169**, 2788–2796.

Keir, M. E., Stoddart, C. A., Linquist-Stepps, V., Moreno, M. E., and McCune, J. M. (2002b). IFN-α secretion by type 2 predendritic cells up-regulates MHC Class I in the HIV-1-infected thymus. *J. Immunol.* **168**, 325–331.

Keppler, O. T., Yonemoto, W., Welte, F. J., Patton, K. S., Iacovides, D., Atchison, R. E., Ngo, T., Hirschberg, D. L., Speck, R. F., and Goldsmith, M. A. (2001). Susceptibility of rat-derived cells to replication by human immunodeficiency virus type 1. *J. Virol.* **75**, 8063–8073.

Keppler, O. T., Welte, F. J., Ngo, T. A., Chin, P. S., Patton, K. S., Tsou, C. L., Abbey, N. W., Sharkey, M. E., Grant, R. M., You, Y., Scarborough, J. D., Ellmeier, W., *et al.* (2002). Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J. Exp. Med.* **195**, 719–736.

Keppler, O. T., Allespach, I., Schuller, L., Fenard, D., Greene, W. C., and Fackler, O. T. (2005). Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor *Nef*. *J. Virol.* **79**, 1655–1665.

Keswani, S. C., Jack, C., Zhou, C., and Hoke, A. (2006). Establishment of a rodent model of HIV-associated sensory neuropathy. *J. Neurosci.* **26**, 10299–10304.

Kim, B. O., Liu, Y., Ruan, Y., Xu, Z. C., Schantz, L., and He, J. J. (2003). Neuropathologies in the transgenic mice expressing human immunodeficiency virus type 1 *Tat* protein under the regulation of the astrocyte-specific glial fibrillary acidic protein promoter and doxcycline. *Am. J. Pathol.* **162**, 1693–1707.

Kitchen, S. G., and Zack, J. A. (1997). CXCR4 expression during lymphopoiesis: Implications for human immunodeficiency virus type 1 infection of the thymus. *J. Virol.* **71**(9), 6928–6934.

Kitchen, S. G., and Zack, J. A. (1999). Distribution of the human immunodeficiency virus coreceptors CXCR4 and CCR4 in fetal lymphoid organs: Implications for pathogenesis in utero. *AIDS Res. Hum. Retroviruses* **15**(2), 143–148.

Koka, P. S., Fraser, J. K., Bryson, Y., Bristol, G. C., Aldrovandi, G. M., Daar, E. S., and Zack, J. S. (1998). Human immunodeficiency virus inhibits multilineage hematopoiesis *in vivo*. *J. Virol.* **72**, 5121–5127.

Koup, R. A., Safrit, J. T., Weir, R., and Gauduin, M. C. (1996). Defining antibody protection against HIV-1 transmission in hu-PBL-SCID mice. *Semin. Immunol.* **8**, 263–268.

Kovalev, G., Duus, K., Wang, L., Lee, R., Bonyhadi, M., Ho, D. D., McCune, J. M., Kaneshim, H., and Su, L. (1999). Induction of MHC Class I expression on immature thymocytes in HIV-1 infected SCID-hu Thy/Liv mice: Evidence of indirect mechanisms. *J. Immunol.* **162**, 7555–7562.

Kulaga, H., Folks, T. M., Rutledge, R., and Kindt, T. J. (1988). Infection of rabbit T-cell and macrophage lines with human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **85**, 4455–4459.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.

Lapenta, C., Santini, S. M., Proietti, E., Rizza, P., Logozzi, M., Spada, M., Parlato, S., Fais, S., Pitha, P. M., and Berlardelli, F. (1999). Type I interferon is a powerful inhibitor of *in vivo* HIV-1 infection and preserves human CD4(+) T cells from virus-induced depletion in SCID mice transplanted with human cells. *Virology* **263**, 78–88.

Lapenta, C., Santini, S. M., Logozzi, M., Spada, M., Andreotti, M., Di Pucchio, T., Parlato, S., and Belardelli, F. (2003). Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-α. *J. Exp. Med.* **198**, 361–367.

Leno, M., Hague, B. F., Teller, R., and Kindt, T. J. (1995). HIV-1 mediates rapid apoptosis of lymphocytes from human CD4 transgenic but not normal rabbits. *Virology* **213**, 450–454.

Leonard, J. M., Abramczuk, J., Pezen, J. W., Rutledge, D. S., Belcher, R., Hakim, J. H., Shearer, G., Lamperth, L., Travis, W., Fredrickson, T., Notkins, A. L., and Martin, M. A. (1988). Development of disease and virus recovery in transgenic mice containing HIV proviral DNA. *Science* **242**, 1665–1670.

Liao, F. G., Alkhatib, G., Peden, K. W., Sharma, G., Berger, E. A., and Farber, F. M. (1997). STRL33, a novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J. Exp. Med.* **185**, 2015–2023.

Lindemann, D., Wilhelm, R., Renard, P., Althage, A., Zinkernagel, R., and Mous, J. (1994). Severe immunodeficiency associated with a human immunodeficiency virus 1 *NEF*/3′-long terminal repeat transgene. *J. Exp. Med.* **179**, 797–807.

Loetscher, D. F., Amara, D. F., Oberlin, D. F., Brass, D. F., and Legler, D. F. (1997). TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. *Curr. Biol.* **7**, 652–660.

Lores, P., Boucher, V., MacKay, C., Pla, M., Von Boehmer, H., Jami, J., Barre-Sinoussi, F., and Weill, J. C. (1992). Expression of human CD4 in transgenic mice does not confer sensitivity to human immunodeficiency virus infection. *AIDS Res. Hum. Retroviruses* **8**, 2063–2071.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., and Axel, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* **47**, 333–348.

Malynn, B. A., Blackwell, T. K., Fulop, G. M., Rathbun, G. A., Furley, A. J., Ferrier, P., Heinke, L. B., Phillips, R. A., Yancopoulos, G. D., and Alt, F. W. (1988). The SCID defect affects the final step of the immunoglobulin VDJ recombinase mechanism. *Cell* **54**, 453–460.

Mariani, R., Rutter, G., Harris, M. E., Hope, T. J., Krausslich, H.-G., and Landau, N. R. (2000). A block to human immunodeficiency virus type 1 assembly in murine cells. *J. Virol.* **74**, 3859–3870.

Mariani, R., Rasala, B. A., Rutter, G., Wiegers, K., Brandt, S. M., Krausslich, H. G., and Landau, N. R. (2001). Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. *J. Virol.* **75**, 3141–3151.

McCune, J. M. (1996). Development and applications of the SCID-hu mouse model. *Semin. Immunol.* **8**, 187–196.

McCune, J. M., Namikawa, R., Kaneshima, H., Shultz, L. D., Lieberman, M., and Weissman, I. L. (1988). The SCID-hu mouse: Murine model for the analysis of human hematolymphoid differentiation and function. *Science* **241**, 1632–1639.

McKinney, D. M., Lewinsohn, D. A., Riddel, S. R., Greenberg, P. D., and Mosier, D. E. (1999). The antiviral activity of HIV-specific CD8+ CTL clones is limited by elimination due to encounter with HIV-infected targets. *J. Immunol.* **163**, 861–867.

Meissner, E. G., Duus, K., Gao, F., Yu, X. F., and Su, L. (2004). Characterization of a thymus tropic HIV-1 isolate from a rapid progressor: Role of the envelope. *Virology* **328**, 74–88.

Mizrachi, Y., Sternas, L., and Volsky, D. J. (1992). The establishment of rodent cell lines persistently producing HIV-1. *Virology* **186**, 167–174.

Moosmayer, D., Reil, H., Ausmeier, M., Scharf, J. G., Hauser, H., Jentsch, K. D., and Hunsmann, G. (1991). Expression and frameshift but extremely inefficient proteolytic processing of the HIV-1 gag and pol gene products in stably transfected rodent cell lines. *Virology* **183**, 215–224.

Mosier, D. E., Guliza, R. J., Baird, S. M., and Wilson, D. B. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* **335**, 256–259.

Mosier, D. E. (1996). Viral pathogenesis in hu-PBL-SCID mice. *Semin. Immunol.* **8**, 255–262.

Mosier, D. E., Gulizia, R. J., Baird, S. M., Wilson, D. B., Spector, D. H., and Spector, S. A. (1991). Human immunodeficiency virus infection of human-PBL-SICD mice. *Science* **251**, 791–794.

Mosier, D. E., Gulizia, R. J., MacIsaac, P. D., Torbett, B. E., and Levy, J. A. (1993a). Rapid loss of $CD4^+$ T cells in human-PBL-SCID mice by noncytopathic HIV isolates. *Science* **260**, 689–692.

Mosier, D. E., Gulizia, R. J., MacIsaac, P. D., Corey, L., and Greenberg, P. D. (1993b). Resistance to human immunodeficiency virus 1 infection of SCID mice reconstituted with peripheral blood leukocytes from donors vaccinated with vaccinia gp160 and recombinant gp160. *Proc. Natl. Acad. Sci. USA* **90**, 2443–2447.

Namikawa, R., Mizuno, T., Matsuoka, H., Jukami, H., Ueda, R., Itoh, G., Matsuyama, M., and Takahashi, T. (1986). Ontogenic development of T and B cells and non-lymphoid cells in the white pulp of the human spleen. *Immunology* **57**, 61–69.

Okamoto, Y., Eda, Y., Ogura, A., Shibata, S., Amagai, T., Katsura, Y., Asano, T., Kimachi, K., Makizumi, K., and Honda, M. (1998). In SCID-hu mice, passive transfer of a humanized antibody prevents infection and atrophic change of medulla in human thymic implant due to intravenous inoculation of primary HIV-1 isolate. *J. Immunol.* **160**, 69–76.

Olivieri, K., Scoggins, R. M., Yeou-cherng, B., Matthews, A., Mark, D., Taylor, J. R., Jr., Chernauskas, D., Hammarskjold, M. L., Rekosh, D., and Camerini, D. (2007). The envelope gene is a cytopathic determinant of CCR5 tropic HIV-1. *Virology* **358**, 23–38.

Osiecki, K., Xie, L., Zheng, J. H., Squires, R., Pettoello-Mantovani, M., and Goldstein, H. (2005). Identification of GM-CSF-stimulating factor and LPS-induced signal transduction pathways that synergize to stimulate HIV-1 production by monocytes from HIV-1 transgenic mice. *AIDS Res. Hum. Retroviruses* **21**, 125–139.

Owman, C. A., Garzino-Demo, A., Cocchi, F., Popovic, M., Sabirsh, A., and Gallo, R. C. (1998). The leukotriene B4 receptor functions as a novel type of coreceptor mediating entry of primary HIV-1 isolates into CD4-positive cells. *Proc. Natl. Acad. Sci. USA* **95**, 9530–9534.

Parren, P., Ditzel, H., Gulizia, R., Binley, J., Barbas, C. I., Burton, D., and Mosier, D. E. (1995). Protection against HIV-1 infection in hu-PBL-SCID mice by passive immunization with a neutralizing human monoclonal antibody against the gp120 CD4-binding site. *AIDS* **9**, 1–6.

Paul, J. B., Wang, E. J., Pettoello-Mantovani, M., Raker, C., Yurasov, S., Goldsten, M. M., Horner, J. W., Chan, J., and Goldstein, H. (2000). Mice transgenic for monocyte-tropic HIV type 1 produce infectious virus and display plasma viremia: A new *in vivo* system for studying the postintegration phase of HIV replication. *AIDS Res. Hum. Retroviruses* **16**, 481–492.

Picchio, G. R., Valdez, H., Sabbe, R., Landay, A. L., Kuritzkes, D. R., Lederman, M. M., and Mosier, D. E. (2000). Altered viral fitness of HIV-1 following failure of protease inhibitor-based therapy. *J. Acquir. Immune Defic. Syndr.* **25**, 289–295.

Pleskoff, O. C., Treboute, A., Brelot, A., Heveker, N., Seman, M., and Alizon, M. (1997). Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **276**, 1874–1878.

Poignard, P., Sabbe, R., Picchio, G. R., Wang, M., Gulizia, R. J., Katinger, H., Parren, P. W., Mosier, D. E., and Burton, D. R. (1999). Neutralizing antibodies have limited effects on the control of established HIV-1 infection *in vivo*. *Immunity* **10**, 431–438.

Reinhardt, B., Torbett, B. E., Gulizia, R. J., Reinhardt, P. P., Spector, S. A., and Mosier, D. E. (1994). Human immunodeficiency virus type 1 infection of neonatal severe combined immunodeficient mice xenografted with human cord blood cells. *AIDS Res. Hum. Retroviruses* **10**, 131–141.

Rizza, P., Santini, S. M., Logozzi, M. A., Lapenta, C., Sestili, P., Gherardi, G., Lande, R., Spada, M., Parlato, S., Belardelli, F., and Fais, S. (1996). T-cell dysfunctions in hu-PBL-SCID mice infected with human immunodeficiency virus (HIV) shortly after reconstitution: *In vivo* effects of HIV on highly activated human immune cells. *J. Virol.* **70**, 7958–7964.

Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G., Doranz, B. J., Parmentier, M., and Doms, R. W. (1997). Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J. Virol.* **71**, 8999–9007.

Safrit, J. T., Fung, M. S., Andres, C. A., Braun, D. G., Sun, W. N., Chang, T. W., and Koup, R. A. (1993). hu-PBL-SCID mice can be protected from HIV-1 infection by passive transfer of monoclonal antibody to the principal neutralizing determinant of envelope gp120. *AIDS* **7**, 15–21.

Samson, M., Labbe, O., Mollereau, C., Vassert, G., and Parmentier, M. (1996). Molecular cloning and functional expression of a new human C-C-chemokine receptor gene. *Biochemistry* **35**, 3362–3367.

Santini, S. M., Lapenta, C., Logozzi, M., Parlato, S., Spada, M., Di Pucchio, T., and Belardelli, F. (2000). Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity *in vitro* and in hu-PBL-SCID mice. *J. Exp. Med.* **191**, 1777–1788.

Santoro, T. J., Bryant, J. L., Pellicoro, J., Klotman, M. E., Kopp, J. B., Bruggeman, L. A., Franks, R. R., Notkins, A. L., and Klotman, P. E. (1994). Growth failure in AIDS-like cachexia syndrome in HIV-1 transgenic mice. *Virology* **201**, 147–151.

Schuler, W., Weiler, I. J., Schuler, A., Phillips, R. A., Rosenberg, N., Mak, T. W., Kearney, J. F., Perry, R. P., and Bosma, M. J. (1996). Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. *Cell* **46**, 963–972.

Schulz, R., and Mellor, A. L. (1996). Self major histocompatibility complex class I antigens expressed solely in lymphoid cells do not induce tolerance in the $CD4^+$ T cell compartment. *J. Exp. Med.* **184**, 1573–1578.

Scoggins, R. M., Taylor, J. R., Jr., Patrie, J., Angelique, B., Van't Wout, A. B., Schuitemaker, H., and Camerini, D. (2000). Pathogenesis of primary R5 human immunodeficiency virus Type 1 clones in SCID-hu mice. *J. Virol.* **74**, 3205–3216.

Shultz, L. D., Schweitzer, P. A., Christianson, S. W., Gott, B., Schweitzer, I. B., McKenna, S., Mobraaten, L., Rajan, V., Greiner, D. L., and Leiter, E. H. (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180–191.

Shultz, L. D., Lang, P. A., Christianson, S. W., Gott, B., Lyons, B., Umeda, S., Leiter, E., Hesselton, R., Wagar, E., Leif, J. H., Kollef, O., Lapidof, T., *et al.* (2000). NOD/LtSz-$Rag1^{null}$ mice: An immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. *J. Immunol.* **164**, 2496–2507.

Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Gillies, S., King, M., Mangada, J., Greiner, D. L., and Handgretinger, R. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2Rnull mice engrafted with mobilized human hematopoietic stem cells. *J. Immunol.* **174**, 6477–6489.

Simpson, R. M., Hubbard, B. S., Zhao, T. M., and Kindt, T. J. (1997). Experimental perinatal transmission of human immunodeficiency virus type 1 by passage of infected T cells. *J. Infect. Dis.* **175**, 1337–1343.

Skowronski, J., Parks, D., and Mariani, R. (1993). Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. *EMBO J.* **12**, 703–713.

Snyder, B. W., Vitale, J., Milos, P., Gosselin, J., Gillespie, F., Ebert, K., Hague, B. F., Kindt, T. J., Wadsworth, S., and Leibowitz, P. (1995). Development and tissue-specific expression of human CD4 in transgenic rabbits. *Mol. Reprod. Dev.* **40**, 419–428.

Speck, R. F., Penn, M. L., Wimmer, J., Esser, U., Hague, B. F., Kindt, T. J., Atchison, R. E., and Goldsmith, M. A. (1998). Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. *J. Virol.* **72**, 5728–5734.

Stanley, S. K., McCune, J. M., Kaneshima, H., Justement, J. S., Sullivan, M., Boone, E., Baseler, M., Adelsberger, J., Bonyhadi, M., Orenstein, J., Fox, C., and Fauci, A. (1993). Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. *J. Exp. Med.* **178**, 1151–1163.

Su, L., Kaneshima, H., Bonyhadi, M., Salimi, S., Kraft, D., Rabin, L., and McCune, J. M. (1995). HIV-1 induced thymocyte depletion is associated with indirect cytopathogenicity and infection of progenitor cells *in vivo*. *Immunity* **2**, 25–36.

Su, L., Kaneshima, H., Bonyhadi, M. L., Lee, R., Auten, J., Du, B., Rabin, L., Hahn, B. H., Terwilliger, E., and McCune, J. M. (1997). Identification of HIV-1 determinants for replication *in vivo*. *Virology* **227**, 45–52.

Sun, J., Soos, T., KewalRamani, V., Osiecki, K., Xie, L., Zheng, J. H., Falkin, L., Santambrogio, L., Littman, D. R., and Goldstein, H. (2006). CD4 specific transgenic expression of human cyclin T1 markedly increases HIV-1 production by CD4+ lymphocytes and myeloid cells in mice transgenic for a provirus encoding a monocyte-tropic HIV-1 isolate. *J. Virol.* **80**, 1850–1862.

Tary-Lehmann, M., and Saxon, A. (1992). Human mature T cells that are anergic *in vivo* prevail in SCID mice reconstituted with human peripheral blood. *J. Exp. Med.* **175**, 503–516.

Taylor, J. R., Kimbrell, K. C., Scoggins, R., Delaney, M., Wu, L., and Camerini, D. (2001). Expression and function of chemokine receptors on human thymocytes: Implications for infection by human immunodeficiency virus type 1. *J. Virol.* **75**, 8752–8760.

Tinkle, B. T., Ueda, H., Ngo, L., Luciw, P. A., Shaw, K., Rosen, C. A., and Jay, G. (1997). Transgenic dissection of HIV genes involved in lymphoid depletion. *J. Clin. Invest.* **100**, 32–39.

Toggas, S. M., Masliah, E., Rockenstein, E. M., Rall, G. F., Abraham, C. D., and Mucke, L. (1994). Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature* **367**, 188–193.

Touraine, J. L. (1983). Bone-marrow and fetal-liver transplantation in immunodeficiencies and inborn errors of metabolism. *Immunol. Rev.* **71**, 103–121.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., and Manz, M. G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**, 104–107.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Chen-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184–187.

Vandekerckhove, B. A., Baccala, R., Jones, D., Kono, D. H., Thefilopoulos, A. N., and Roncarolo, M. G. (1992a). Thymic selection of the human T cell receptor V beta repertoire in SCID-hu mice. *J. Exp. Med.* **176**(6), 1619–1624.

Vandekerckhove, B. A., Namikawa, R., Bacchetta, R., and Roncarolo, M. G. (1992b). Human hematopoietic cells and thymic epithelial cells induce tolerance via different mechanisms in the SCID-hu mouse thymus. *J. Exp. Med.* **175**(4), 1033–1043.

Van Kuyk, R., Torbett, B. E., Gulizia, R. J., Leath, S., Mosier, D. E., and Koenig, S. (1994). Cloned human Cd8+ cytotoxic T lymphocytes protect human peripheral blood leukocyte-severe combined immunodeficient mice from HIV-1 infection by an HLA-unrestricted mechanism. *J. Immunol.* **153**, 4826–4833.

Vieillard, V., Jouveshomme, S., Leflour, N., Jean-Pierre, E., Debre, P., De Maeyer, E., and Autran, B. (1999). Transfer of human CD4(+) T lymphocytes producing beta interferon in hu-PBL-SCID mice controls human immunodeficiency virus infection. *J. Virol.* **73**, 10281–10288.

Wang, E. F., Sun, J., Pettoello-Mantovani, M., Anderson, C. M., Osiecki, K., Zhao, M. L., Lopez, L., Lee, S. C., Berman, J. W., and Goldstein, H. (2003). Microglia from mice transgenic for a provirus encoding a monocyte-tropic HIV type 1 isolate produce infectious virus and display *in vitro* and *in vivo* upregulation of lipopolysaccharide-induced chemokine gene expression. *AIDS Res. Hum. Retroviruses* **19**, 755–765.

Watanabe, S., Terashima, K., Ohta, S., Horibata, S., Yajima, M., Shiozawa, Y., Dewan, M. Z., Yu, Z., Ito, M., Morio, T., Shimizu, N., Honda, M., *et al.* (2007). Hematopoietic stem cell-engrafted NOD/SCID/IL2rγ^{null} mice develop human lymphoid system and induced long-lasting HIV-1 infection with specific humoral immune responses. *Blood* **109**, 212–218.

Weng, X., Priceputu, E., Chrobak, P., Poudrier, J., Kay, D. G., Hanna, Z., Mak, T. W., and Jolicoeur, P. (2004). $CD4^+$ T cells from CD4C/HIV *Nef* transgenic mice show enhanced activation *in vivo* with impaired proliferation *in vitro* but are dispensable for the development of a severe AIDS-like disease. *J. Virol.* **78**, 797–807.

Yamamura, Y., Kotani, M., Chowdhury, M. I. H., Yamamoto, N., Yamaguchi, K., Karasuyama, H., Katsura, Y., and Miyasaka, M. (1991). Infection of human $CD4^+$ rabbit cells with HIV-1: The possibility of the rabbit as a model for HIV-1 infection. *Int. Immunol.* **3**, 1183–1187.

Yasuda, J., Miyao, T., Kamata, M., Aida, Y., and Iwakura, Y. (2001). T cell apoptosis causes peripheral T cell depletion in mice transgenic for the HIV-1 vpr gene. *Virology* **285**(2), 181–192.

Yoshida, A., Tanaka, R., Murakami, T., Takahashi, Y., Koyanagi, Y., Nakamura, M., Ito, M., Yamamoto, N., and Tanaka, Y. (2003). Induction of protective immune responses against R5 human immunodeficiency virus type 1 (HIV-1) infection in hu-PBL-SCID mice by intrasplenic immunization with HIV-1 pulsed dendritic cells: Possible involvement of a novel factor of human CD4(+) T-cell origin. *J. Virol.* **77**, 8719–8728.

Yoshida, A., Tanaka, R., Kodama, A., Yamamto, N., Ansari, A. A., and Tanaka, Y. (2005). Identification of HIV-1 epitopes that induce the synthesis of a R5 HIV-1 suppression factor by human $CD4^+$ T cells isolated form HIV-1 immunized hu-PBL SCID mice. *Clin. Dev. Immunol.* **12**, 235–242.

Zhang, Y. J., Dragic, T., Cao, Y., Kostrikis, L., Kwon, D. S., Littman, D. R., KewalRamani, V. N., and Moore, J. P. (1998). Use of coreceptors other than CCR5 by non-syncytium-inducing adult and pediatric isolates of human immunodeficiency virus type 1 is rare *in vitro*. *J. Virol.* **72**, 9337–9344.

Zhang, L., He, T., Huang, Y., Chen, Z., Guo, Y., Wu, S., Kunstman, K. J., Brown, R. C., Phair, J. P., Neumann, A. U., Ho, D. D., and Wolinsky, S. M. (1998). Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. *J. Virol.* **72**, 9307–9312.

Zheng, Y. H., Yu, H. F., and Peterlin, B. M. (2003). Human p32 protein relieves a post-transcriptional block to HIV replication in murine cells. *Nat. Cell Biol.* **5**, 611–618.

Marco Schiavone, Ileana Quinto, and Giuseppe Scala

Department of Clinical and Experimental Medicine, University of Catanzaro, Catanzaro, Italy

Perspectives for a Protective HIV-1 Vaccine

I. Chapter Overview

A major goal of the world public health is the development of an effective *Human immunodeficiency virus 1* (HIV-1) vaccine to counteract the AIDS epidemic in geographic regions, mostly African, such as Zimbabwe and South Africa, characterized by degraded social and economic conditions. In these countries, the AIDS epidemic is a major obstacle toward any improvement of the social and economic expectancies. In fact, the high rate of infections among young people has resulted in dramatic decrease of the lifetime expectancy, increased number of single parent families, millions of orphans, and a substantial reduction of the gross national product (GNP);

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56014-X

this further decreases the economic resources allocated to public health programs, a situation that in turn perpetuates the social conditions that favors the spreading of AIDS. As a diffuse availability of drugs for an effective antiretroviral therapy is not in sight, a prophylactic vaccine remains the most reliable solution. The development of an effective vaccine has encountered several problems associated with the unique characteristics of the envelope structure and of the large degree of viral variability. However, recent advance in understanding the quaternary structure of the viral envelope and of the immune correlates of protection have fostered several studies based on a rational design of immunogenic epitopes. When accomplished, these studies may lead to an effective HIV vaccine.

II. Introduction

The continuous spreading of the HIV-1 infection worldwide is a major health problem with an estimated 39.4 million of subjects infected at the end of 2004 (WHO/UNAIDS report). This burden is increasing at the annual rate of 5 million new infections, most of them in underdeveloped countries, particularly in sub-Saharan Africa where effective prevention measures and clinical treatments are either spotty or not available. This grim picture calls for the development of an effective vaccine against HIV-1 to counteract the devastating individual and societal outcomes of the HIV-1 pandemic. However, efforts to develop a protective HIV-1 vaccine have been hindered by difficulties in identifying T- and B-cell epitopes capable of inducing a broad and sustained immune response. In the next sections, we review the life cycle of HIV-1 and the natural history of HIV-1 infection, address the major problems encountered in the quest for the HIV-1 vaccine, and discuss the main approaches toward the development of an effective HIV-1 vaccine. Finally, we explore recent advance in understanding the physical structure of the viral envelope and the virus interplay with the host immune system, which may shed some optimism on the development of an effective vaccine.

A. Natural History of HIV-1 Infection

HIV-1 and its simian counterpart *Simian immunodeficiency virus* (SIV) are lentivirus of the large Retroviridae family characterized by a long incubation time. All retroviruses contain a single-stranded RNA genome that is converted to a double-stranded DNA form (provirus), which integrates into the host cell chromosomal DNA. HIV-1, HIV-2, and SIV are primate lentiviruses (Ratner *et al.*, 1987; Wain-Hobson *et al.*, 1985) that induce an acquired immunodeficiency disease in the infected hosts (Fultz *et al.*, 1986; Novembre *et al.*, 1997).

The HIV-1 proviral genome includes nine open reading frames that encode for 15 viral proteins (Gotte *et al.*, 1999): (1) group-associated antigen (*gag*) encoding structural core proteins, (2) polymerase (*pol*) encoding enzymatic proteins, and (3) envelope (*env*) encoding the receptor-binding protein. The HIV-1 genome codes for two regulatory proteins (Tat and Rev) and four accessory proteins (Nef, Vif, Vpu, and Vpr) that are required for efficient virion replication (Fig. 1). Two long-terminal repeat (LTRs) flank both the 5′ and 3′ ends of the proviral DNA genome; the 5′ LTR includes the HIV-1 promoter and enhancer sequences that regulate viral gene expression. The matrix protein (MA or p17) is associated with the virus lipid bilayer (Dorfman *et al.*, 1994) and with the viral capsid (CA or p24) that contains the RNA genome, which is associated with the nucleocapsid proteins (NC or p7/p6). The reverse transcriptase (RT) converts the genomic RNA into

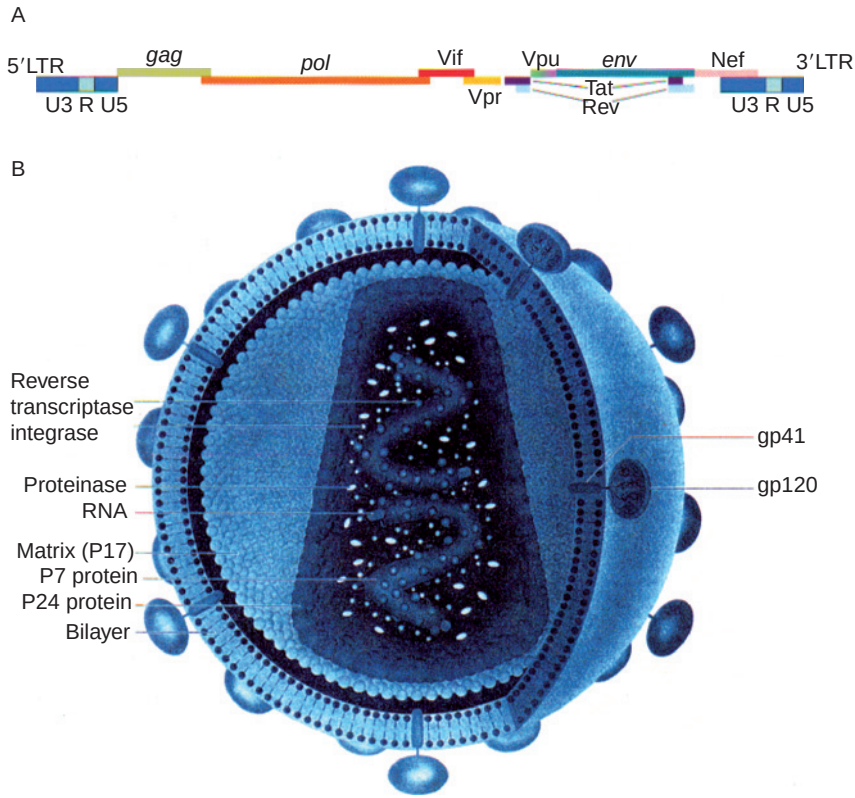


FIGURE 1 (A) Schematic representation of the viral genome containing replication genes (*pol*, *vif*, *nef*, *tat*, *rev*, *vpu*, *vpr*) and assembly genes (*gag*, *env*); (B) HIV-1 physical structure. The glycosylated envelope surrounds the viral matrix that contains the viral genome and enzymes required for replication and assembly of the viral particle.

proviral DNA; integrase (IN) promotes the insertion of the proviral DNA into the host chromosome; and the protease (PR), which is responsible for proteolytic cleavage and activation of the Gag-Pol (p160) polypeptide.

The viral particle binds to $CD4^{+}CCR5R^{+}$ cells by engaging a CD4 amino-terminal domain with the gp120 envelope external domains, a trimeric structure characterized by unique flexibility and heavy glycosylation. This binding results in structural changes that expose a β-sheet structure, which in turn binds to the amino-terminal domain of CCR5, the cell coreceptor (Fig. 2). The virus cell binding will result in the gp41 triggering of virus cell membrane fusion and viral entry. Further steps include retrotranscription and nuclear import of the viral genome, followed by random integration into the host genome. In the infected cell, the integrated virus may establish a long-lasting viral reservoir; upon cell activation stimuli, the integrated virus genome undergoes the gene transcription, viral assembly, cell membrane budding, and viral spread.

As HIV-1 is transmitted both sexually and hematogenously, the infection may start by crossing a mucosal barrier or by infecting peripheral blood T and monocyte/macrophage cells. In this setting, HIV-1 can spread by either free viral particles or as cell-associated virus.

As mentioned earlier, the intrinsically inaccurate viral replication (at least one base substitution/viral cycle), associated with the random recombination of the two RNA/DNA genomes/viral particle during retrotranscription, gives rise to an enormous sequence variability, with each viral

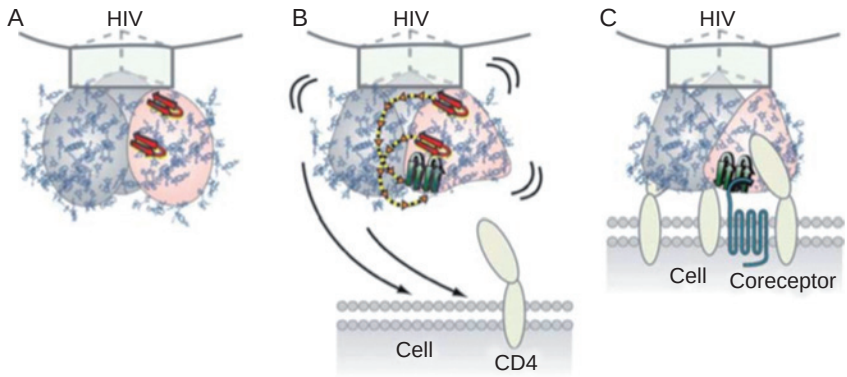


FIGURE 2 Changes in the structure of the HIV envelope on binding to target cells. (A) The heavily glycosylated envelope comprises gp120 and gp41 as a trimer. This structure was solved by Chen *et al.* (2005) for SIV. (B) When the envelope binds to CD4, the gp120 undergoes a conformational change that forms and exposes the binding site for the coreceptor CCR5 or CXCR4 (shown as red β sheet changing to green). (C) This event is followed by conformational changes in gp41 that trigger membranes fusion and virus cell entry (Kwong, 2005). Reprinted by permission from Macmillan Publishers Ltd.: *Nature*, Kwong, 2005 © 2005 Nature Publishing Group.

particle differing from the original infectious virion. This large sequence variability has driven a rapid and ongoing viral evolution where the nitial infectious virus has evolved in distinct clades and quasispecies. Indeed, HIV-1 is genotypically divided into three distinct groups: major (M), outlier (O), and non-M non-O (N) with the majority of HIV-1 strains comprising the M group. Since its introduction into the human population, the M group has evolved into at least 10 distinct subtypes and 13 different circulating recombinant forms (CRFs) (Louwagie *et al.*, 1993; Myers, 1994).

B. Immune Response Elicited During HIV-1 Infection

HIV-1 is transmitted by sexual intercourse, blood products, contaminated needles, or from mother-to-child during the perinatal period (Curran *et al.*, 1985). High levels of plasma viremia in the range of 10^6 plasma RNA viral copies can be detected at around weeks 4–6 from the putative time of infection (Fonteneau *et al.*, 2004). This acute infection is followed by a long-term chronic infection characterized by significant decline of viral loads (10^3–10^5 copies/ml) that may remain stable for several months (Fig. 3).

In early infection, innate immunity mediated by activated DC and NK cells are detected at the very first days of viremia (Alter *et al.*, 2004; Bhardwaj, 2001; Fonteneau *et al.*, 2004; Pulendran *et al.*, 2001) and is associated with rising levels of plasma IFN-γ (Fonteneau *et al.*, 2004). The adaptive immune responses can be detected at around week 4 and includes a strong CD8 T-cell response that is probably responsible for the significant reduction of the viral load (Borrow *et al.*, 1994; Wilson *et al.*, 2000), as indicated by the evidence that depletion of $CD8^+$ cells by anti-CD8 antibody infusion during primary SIV infection in macaques results in sustained high viremia (Schmitz *et al.*, 1999). Further evidence for a role of CD8 T cells

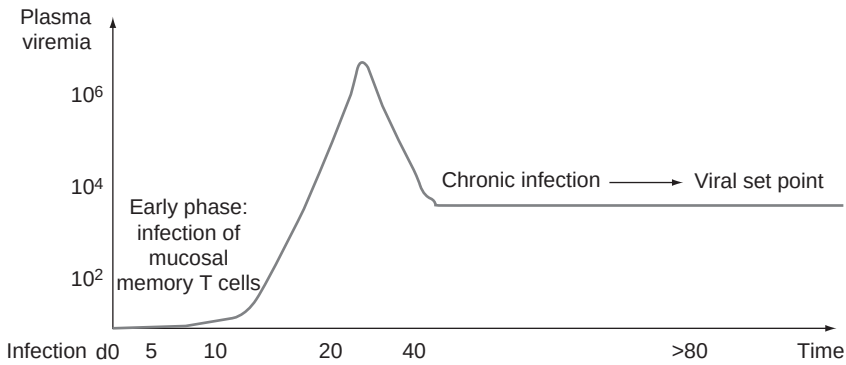


FIGURE 3 The natural history of HIV-1 infection. The change in virus load is shown over time as the virus spreads.

comes from studies in macaques and humans where escape mutations in immunodominant epitopes recognized by $CD8^+$ T cells are selected during primary HIV infection (Borrow *et al.*, 1997; Evans *et al.*, 1999; Jones *et al.*, 2004; Price *et al.*, 1997). A $CD4^+$ T-cell response is also detectable during the primary infection, and may provide T help to $CD8^+$ effector T cells; however, this function is undermined by the susceptibility of $CD4^+CCR5^+$ memory to HIV-1 infection (Amyes *et al.*, 2003; Douek *et al.*, 2002; Mattapallil *et al.*, 2005; Pitcher *et al.*, 1999). Further, T cells from infected individuals show variable expression of perforin, granzymes A and B, IFN-γ, IL-2, MIP-1α, MIP-1β, and RANTES (Appay *et al.*, 2000; Champagne *et al.*, 2001); however, the contribution of these phenotypes to viral control and disease progression is controversial.

Neutralizing antibodies appear at months 2–3 postinfection (Safrit *et al.*, 1994) at the time of chronic infection. This antibody response does not neutralize endogenous virus variants (Derdeyn *et al.*, 2004) and may not play a significant role in the course of acute infection. However, the antibodies exert a strong antiviral force, as shown by the selection of the escape variants, and may contribute to the control of viremia.

C. Requirements for an Effective HIV-1 Vaccine

As detailed above, an effective HIV vaccine should take into account the unique features of HIV-1 infection:

a. Infection can start by crossing the mucosal barrier and hematogenously as free viral particle and virus-infected T cell or monocyte/macrophage lineage cells;
b. Extreme variability of the envelope proteins that include distinct clades and recombinant strains;
c. Complex envelope structure that provides little accessibility to antibody neutralization sensitive epitopes; and
d. As there is no record of viral clearance in HIV-infected subjects, a prophylactic vaccine should block the primary infection at the mucosal and hematogeneous level.

In this regard, an effective vaccine-induced immune response should elicit neutralizing antibodies to the exposed oligomeric envelope of HIV-1. Indeed, passively transferred neutralizing antibodies have provided true sterilizing protection in rhesus macaques from mucosal and systemic infection with HIV-1 strains (Gauduin *et al.*, 1997; Mascola *et al.*, 2000; Parren *et al.*, 2001). By contrast, once infected $CD4^+CCR5^+$ memory T cells have started the infection, a vaccine eliciting a cellular immune response would destroy HIV-1-infected cells before the release of new virus particles (Van Baalen *et al.*, 1998) and may function in synergy with the neutralizing antibodies to limit early spread of infection. In support of this possibility, a small number of

highly HIV-1-exposed and -uninfected people have been identified that show variable levels of HIV-1-specific $CD8^+$ T-cell and/or $CD4^+$ T-cell responses (Clerici *et al.*, 1994; Goh *et al.*, 1999; Hladik *et al.*, 2003; Rowland-Jones *et al.*, 1995, 1998). Consistently, in these subjects HIV-1-specific T cells have been found in cervical mucosal lavage samples (Kaul *et al.*, 2000). One study of exposed but uninfected sex workers found that later reduction in exposure to HIV actually increased susceptibility to infection (Kaul *et al.*, 2001), suggesting that protection from systemic infection requires the multiple boosting of low-grade viral exposure. Unfortunately, the immune mechanisms underlying the observed protection have not been identified; the exposed uninfected subjects may be infected with HIV-1 at a low level, for example, in gut-associated lymphoid tissue (GALT) (Zhu *et al.*, 2003) and be protected from systemic infection by retroviral interference, by a genetic background that would limit HIV-1 entry and/or virus production, as in the case of CCR5/Δ32 allele or of yet unknown gene mutations that interfere with virus postentry steps, such as APOBEC3G (Holmes *et al.*, 2007).

HIV-1 is highly variable with a considerable heterogeneity of virus RNA and protein sequences (Korber *et al.*, 2000). There are three major types of HIV-1, M, N, and O, which represent separate introductions into humans from chimpanzees. The M type includes six main subtypes, or clades, A, B, C, D, E, and G, that represent early branching of the virus evolution in human subjects (Korber *et al.*, 2000). The clades differ by 20% of amino acid sequence and have distinct geographical distributions (Fig. 4.) Despite this huge heterogeneity, consensus sequences for each clade have been identified, suggesting an evolution toward conservation. This may allow the selection shared epitopes by matching the vaccine sequences and virus clades to focus the T-cell response toward conserved epitopes. An additional constraint for a T-cell-focused vaccine is coming from the evidence that T-cell response is driven toward few immunodominant epitopes that would likely result in escape mutant virus particles (Goulder *et al.*, 1997; Yu *et al.*, 2002). Immunodominant epitopes are also selected during antigen processing according to their affinity constants for a given HLA molecule (Yewdell and Del Val, 2004).

III. Current Strategies in Developing an HIV-1 Vaccine

A. Live-Attenuated HIV

The rationale for using attenuated virus for vaccine approached stems from the historical use of live-attenuated viruses to control viral infections, such as measles, rubella, smallpox, and mumps (Dorner and Barrett, 1999; Jones, 2002). These vaccines have been shown to be effective in lowering or preventing the natural infection by stimulating both a cell-mediated and

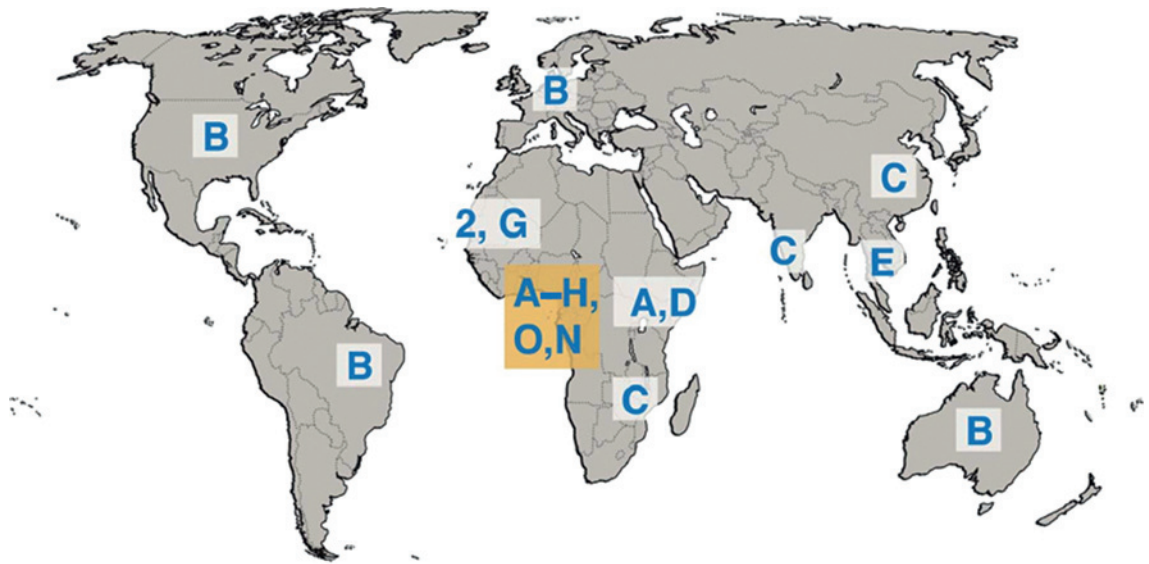


FIGURE 4 Worldwide distribution of the major HIV-1 subtypes (clades) and HIV-2. The subtypes A–H are subdivisions of the M strain; the O and N strains are very close to SIV strains.

humoral immune responses. Soon after the global emergence of the HIV-1 infection, several strains of SIV or SHIV have been evaluated in monkeys as vaccines. These studies were fostered by a unique report of the lack of pathogenicity of live-attenuated HIV infection in a cohort of individuals [The Sydney Blood Bank Cohort (SBBC)] infected with live-attenuated strain(s) of HIV (1981–1984) from a common infected donor (Learmont *et al.*, 1992; Rhodes *et al.*, 1999). Nine subjects were infected with an attenuated strain of HIV-1, which contained a 3′ end deletion of the *nef* gene (Deacon *et al.*, 1995). These patients maintained stable $CD4^+$ T-cell levels, possibly indicating that the Δnef HIV was not pathogenic. Three of the seven members of SBBC were long-term non-progressors (LTNP) with near normal $CD4^+$ T-lymphocyte counts and showed mild HIV/AIDS symptoms (Dyer *et al.*, 1999; Learmont *et al.*, 1992; McIntyre *et al.*, 1999; Zaunders *et al.*, 1999). Three members died of non-AIDS-related issues. Each LTNP maintained a strong HIV-specific proliferative and CTL responses to Gag_{p24}. Two recipients and the original donor showed a significant decline of $CD4^+$ T-cell counts, undetectable viral loads, and were termed long-term survivors (LTS). Two of the LTS members (D36 and C98) were reevaluated in 2002 before starting the HAART therapy (Birch *et al.*, 2001) and showed low $CD4^+$ T-cell counts and high viral loads. After the initial month of HAART, both individuals experienced a significant drop in the level of viremia and an increase in the number of $CD4^+$ T lymphocytes. By sequencing of virus samples from both members, the deletion in the overlapping region between *nef*/LTR was found to be larger than the original documented deletion. In 1999, the donor developed AIDS, and the viral strains were collected before (1995) and after (1999) the onset of AIDS (Jekle *et al.*, 2002). Both isolates were less effective in depleting $CD4^+$ T cells compared with a reference dual tropic strain containing the *nef* gene. However, the virus isolated in 1999 induced a stronger apoptosis in $CD4^+$ T cells as compared with the 1995 sample. Indeed, while the 1995 viral strain was restricted to $CCR5^+$ cells, the 1999 isolate could efficiently infect both $CCR5^+$ and $CXCR4^+$ expressing cells. This conversion from an R5-restricted to an X4-phenotype was correlated with enhanced aggressiveness and anticipated some safety issues and hurdles in exploiting live-attenuated strains as vaccines.

1. Live-Attenuated SIV Vaccines

In initial studies, the T-cell-line adapted SIV1A11 molecular clone was attenuated by the addition of a premature stop codon in the *vpr* gene (Luciw *et al.*, 1998). The rhesus macaques vaccinated by infection with SIV1A11 were not protected from SIV superinfection, while disease progression was delayed (Marthas *et al.*, 1990), indicating that a live-attenuated viral infection could elicit some protective immunity. Further studies focused on abrogating Nef function in SIVmacC8 by deleting 12 base pairs in the *nef*

region overlapping the 5′ LTR (Whatmore *et al.*, 1995). At 17 weeks postvaccination, the vaccine strain reverted to a pathogenic form and the monkeys developed AIDS-like disease. Sequence analysis of virus strains isolated from the vaccinated monkeys contained a functional Nef protein as a result of the restoration of the deleted gene sequences. Similar results were observed in the case of a live-attenuated SIVmac239 vaccine carrying a single base-pair substitution that generated a premature stop at amino acid 93 in Nef (Kestler *et al.*, 1991). These results indicated that future SIV-based live vaccines would require additional attenuation steps to reduce the pathogenicity of the vaccine virus.

To increase the level of attenuation, two attenuated SIV strains, SIVmac293Δ*nef* (SIVΔ*nef*) containing an inactivating deletion of the *nef* gene, and SIVmac293Δ3 (SIVΔ3), included deletion of *nef* and *vpr* genes, together with the negative regulatory element (NRE) of the LTR were generated (Gibbs *et al.*, 1994; Montefiori *et al.*, 1996). These deletions resulted in lower levels of virus replication in the absence of disease. In fact, rhesus macaques vaccinated intravenously with SIVΔnef or SIVΔ3 developed a persistent infection with the vaccine strain and had long-lasting anti-Env and anti-Gag antibodies. Upon challenge with the wild type, pathogenic SIVmac251, vaccinated macaques showed persistent immune responses and tested negative for simian AIDS as compared to naive monkeys. However, a deep analysis revealed that the vaccine caused an AIDS-like illness in adult macaques and subsequently death in neonatal macaques at high doses (Baba *et al.*, 1995, 1999), indicating that a similar live-attenuated HIV-1 vaccine would likely result in AIDS in vaccinated patients.

A further constraint of attenuated lentiviruses comes from the evidence that the degree of attenuation inversely correlates with the ability to elicit effective immune responses. In this regard, Johnson *et al.* (1999) have shown that SIV strains with varying degrees of attenuation differed in their ability to elicit a substantial immune response in rhesus macaques. Female monkeys were vaccinated intravenously with one of the following attenuated SIVmac239 strains: (1) SIVmac239D3, which lacked *nef*, *vpr*, and *nef* sequences that overlap U3 (US); (2) SIVmac239D3X missing *nef*, *vpx*, and US sequences; and (3) SIVmac239D4 lacking *nef*, *vpr*, *vpx*, and US.

The follow-up of SIVΔ3, SIVmac293Δ3X, or SIVmac293Δ4 (SIVΔ4) showed that rhesus macaques vaccinated with any of these three vaccines and then challenged with pathogenic SIVmac251 had low levels of viremia and normal $CD4^+$ cell counts. After challenge, vaccinated monkeys experienced viral loads lower than the challenged naive animals. In this study, the protection from high viremia was ascribed to an early CTL response directed at epitopes in the Gag protein and not to neutralizing antibodies against envelope.

Several reports have shown that live-attenuated SIV vaccines induce cross-protection against SHIV challenge (Kumar *et al.*, 2000; Nilsson *et al.*, 1998). In these studies, rhesus macaques vaccinated with SIVΔ3 or

SIVmac239 showed a significant degree of protection from a viral challenge with the highly pathogenic SHIV89.6PD or pathogenic SIVsmE660 (Abel *et al.*, 2003; Miller and Abel, 2005; Wyand *et al.*, 1999). In further studies, monkeys inoculated with SIVΔ3 and challenged intravenously with SHIV89.6PD were protected from $CD4^+$ T-cell depletion, showed low levels plasma viremia (300–10,000 copies of RNA/ml of plasma) and did not develop an AIDS-like disease. These results were replicated in monkeys vaccinated with SIVΔ3 and challenged with SIVsmE660 (Quinto *et al.*, 2004; Wyand *et al.*, 1994).

2. *Live-Attenuated SIV-HIV (SHIV) Chimeric Vaccines*

Chimeric SIV-HIV (SHIV) viruses carry an envelope from HIV (Enose *et al.*, 2004; Kumar *et al.*, 2002; Warren, 2002; Willey *et al.*, 2003; Yoshino *et al.*, 2000), thus offering the advantage of eliciting an antibody response that may be translated to human subject. The prototypic NM-3rN SHIV encodes the *env*, *tat*, *rev*, *vpu*, and *vpr* gene sequences of $HIV\text{-}1_{NL4\text{-}3}$ and the LTRs, *gag*, *pol*, *vif*, *vpx*, and *nef* genes from SIVmac239 (Kuwata *et al.*, 1995; Sakuragi *et al.*, 1992; Shibata *et al.*, 1991). This strain has been shown to be nonpathogenic for monkeys (Igarashi *et al.*, 1997; Johnson, 2002) and to induce a strong and long-lasting cell-mediated and antibody immune responses (Ui *et al.*, 1999a). Several live-attenuated SHIV vaccines have been developed (Enose *et al.*, 2004; Igarashi *et al.*, 1997; Iida *et al.*, 2004; Kumar *et al.*, 2002; Mackay *et al.*, 2004; Ui *et al.*, 1999b), including SHIV-dn (deleted *nef*), SHIV-drn (deleted *vpr* and *nef*), SHIV-dxrn (deleted *vpx*, *vpr*, and *nef*), SHIV-NI (deleted *nef*, plus IFN-γ expression), and live-attenuated SHIV-4 (deleted *vpu* and *nef*) and $SHIV_{ppc}$ (deleted *vpu*). Results from studies that utilized the above SHIV strains all showed that

a. Vaccinated macaques remained disease-free before challenge.
b. Fifty percent of the macaques immunized with SHIV-dn elicited neutralizing antibodies and CTLs specific for SIV Gag and HIV-1 Env proteins. Moreover, most animals tested positive for natural killer cell activity.
c. Upon intravenous challenge with SHIVNM-3rN, no signs of integrated genome were found in the plasma, PBMCs, or inguinal lymph nodes two years after vaccination. Macaques, immunized with SHIV-dn, were protected from homologous challenge, whereas two of four animals vaccinated with SHIV-drn and SHIV-dxrn (four of four) were infected with the challenge strain.
d. Monkeys vaccinated with SHIV-NI (expressing all the genes of SHIVNM-3rN plus rhesus IFN-γ) showed increases in cytokine production without a reduction in viral replication following challenge.

Considered collectively, live-attenuated vaccines elicit a strong CTL response together with high levels of neutralizing antibodies (Johnson and

Desrosiers, 1998). Moreover, live-attenuated lentiviral vaccines express effectively multiple viral antigens including the native envelope and are capable of infecting and recruiting professional antigen presenting cells (APCs). However, vaccine using live-attenuated lentiviral vaccines has been limited to nonhuman primate studies based on the following evidences: (1) reversion of the attenuated virus to a virulent form, (2) likely recombination of the vaccine strain with pathogenic virus in an infected individual, and (3) ability of the proviral genome to integrate and persist into the host genome. Further, recent reports have shown an inverse relationship between attenuation and efficacy, as observed in rhesus macaque studies. Indeed, the degree of attenuation decreases the efficacy of the vaccines (Denesvre *et al.*, 1995; Johnson, 2002; Johnson *et al.*, 1999).

3. Induction of T-cell Immune Response

A T-cell-inducing vaccine is expected to target the virus at mucosal sites by acting at the early stages of infection and to eliminate the virus. However, there are as yet no reports of SIV vaccination in macaques that have described a clearance of the infection. Nevertheless, consistent evidence from monkeys studies indicates that T-cell-inducing vaccines do limit damage by controlling HIV-1 acute viremia and by lowering the viral set points during the acute phase of infection. In this regard, a T-cell vaccine would result in a long-term, disease-free chronic infection with little loss of immune competence as described in the case of Human T-lymphotropic virus 1 (HTLV-1) and EBV infections. However, this possibility is undermined by the evidence that T-cell response drives virus escape and offers little protection from endogenous strains and from superinfecting viruses (Altfeld *et al.*, 2002).

Numerous vaccine approaches based on CD8 T-cell activity have been validated in monkey models of SIV or SHIV infections. The vaccines have exploited the immune response elicited by *in vivo* expression tools, including plasmid DNA carrying one or more HIV genes, recombinant modified vaccinia virus Ankara (MVA), recombinant adenovirus-5, and recombinant vesicular stomatitis virus, as single vectors or in combination with some of these (Amara *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002). In these experiments, the challenge virus was the pathogenic SIV-HIV hybrid virus SHIV89.6PD that expresses a CXCR4-specific HIV envelope and causes very rapid loss of $CD4^+$ T cells and immunodeficiency (Chen *et al.*, 2001). Results from these studies have shown no sterilizing immunity as vaccinated animals were all infected; however, immunized animals retained substantial levels of peripheral CD4 T cells, and remained healthy with virus loads 1000-times lower than the unvaccinated controls. It is noteworthy that in similar studies where immunized monkeys had been challenged with the CCR5 using viruses SIVmac239 or SIVmac251, the outcomes have shown a less effective control of the infection indicating that a more robust and

strain-specific immune response is required to achieve a clinically relevant degree of protection.

Attempts to translate the results from monkeys studies to human subjects have been more difficult and disappointing. As reported in Table I, several human trials are in progress, with data from at least another five

TABLE I Current Completed HIV Vaccine Clinical Trials[a]

Delivery	*Clade*	*Immunogen*	*Phase*
DNA	A, B, C	e, g, p, n	I
DNA	C	g, p	I
DNA	C	e, g, p, n, t	I
DNA	A, B, C	g, p, n, e	I
DNA	A, B, C	e, g, p, n	I
DNA	A, B, C, D, E	Epitopes	I
DNA	B	G	I
DNA	B	g, p	I
DNA	B	e, g, p, t, r, n	IIA
DNA	A, B, C	e, g, p, n	IIA
DNA + 120	B	E	I
DNA + MVA	A	G	I × 3
DNA + MVA	A	G	IIA × 2
DNA + FWPV	B	e, g, p, r, t, v	IIA
DNA + Ad5	B	G	I
MVA	A	G	I
MVA	C	e, g, p, n, t	I
MVA + FWPV	B	e, g, p, t, r, n	I
CNPV	B	e, g, p	I
CNPV + 120	B, E	e, g, p	III
Ad5	A, B, C	g, p, n	I
Ad5	B	G	I
Ad5 + CNPV	B	g, p	I
Ad5	B	g, p, n	IIB
AAV	C	g, p, n, t	I
VEEV	C	G	I
Protein	D	E	I
Protein	B	T	I
Protein	B	E	I
120	B	E	III
120	E	E	III
VLP	B	e, g	I
VLP	C	E	I

[a]Abbreviations: DNA, plasmid DNA; MVA, modified vaccinia virus Ankara; 120, glycoprotein gp120; FWPV, *fowlpox virus*; CNPV, *canarypox virus*; Ad5, adenovirus-5; Lpep, lipopeptide; AAV, adeno-associated virus; VEEV, *Venezualan equine encephalitis virus*; VLP, virus-like particle; e, HIV-1 envelope; g, HIV-1 gag; p, HIV-1 polymerase; n, HIV-1 nef; t, HIV-1 tat; v, HIV-1 vpr. (Please refer to http://www.iavireport.org/trialsdb/ for more detail.)

trials completed but not available. Most of the results from clinical trials have come from antigens delivered by plasmid DNA, MVA, canarypox, fowlpox, and adenovirus-5, adeno-associated virus (AAV), *Venezuelan equine encephalitis virus*. The expressed HIV proteins have included gag, pol, nef, env, and tat; the subtypes gene sequences from B or C, A, D, and E strains have been tested. The available results are as follows:

1. DNA induces a weak, CD4 T-cell-focused response in humans (MacGregor *et al.*, 2002).
2. The DNA prime followed by MVA boost induces a stronger immune response; $CD4^+$ T-cell response predominates, peaks at day 7 after MVA and is short lived (Vuola *et al.*, 2005).
3. MVA, canarypox, and fowlpox induce a weak primary T-cell response in humans (Edupuganti *et al.*, 2004).
4. MVA can boost $CD8^+$ T-cell responses (McShane *et al.*, 2004).
5. Recombinant, replication defective adenovirus-5 provides a substantial and sustained $CD8^+$ T-cell response in humans (Harro *et al.*, 2005); preexisting antibodies to adenovirus-5 reduce the effectiveness of recombinant adenovirus-5 vaccines (Sumida *et al.*, 2005).

Clearly, results from these approaches have shown the limitations of the currently available vectors and have underscored the low immunogenicity of the expressed viral proteins. However, these studies have shown good levels of safety associated with various vectors and have indicated that the prime-boost regimens induce a stronger immune response.

B. Induction of Antibody-Mediated Immunity

Several studies have shown that neutralizing antibodies with broad specificity can protect from the onset of HIV-1 infection if present at a threshold concentration at the time of the viral inoculum. Indeed, passive administration of broadly neutralizing monoclonal HIV-1 envelope-specific antibodies to macaques shortly before infection with SHIVs carrying an HIV-1 envelope prevents infection (Parren *et al.*, 2001). Similar results have been reported in macaque trials where vaginal administration of neutralizing antibodies before the viral challenge resulted in full protection from systemic infection (Gauduin *et al.*, 1997; Mascola *et al.*, 2000). Further evidence came from mice with severe combined immunodeficiency (SCID) mice that were reconstituted with human lymphoid tissue followed by infection with HIV-1 (Gauduin *et al.*, 1997; Mascola *et al.*, 2000). These experiments also indicated that the antibodies have to be inoculated, and by inference should be elicited, before the virus encounter, as virus escape mutant are quickly selected (Veazey *et al.*, 2003).

The initial optimism regarding the possibility of eliciting neutralizing antibodies by using monomeric gp120 has been thwarted by the negative

results from two VAXGEN phase III efficacy trials in the United States and Thailand (Pitisuttithum *et al.*, 2004).

This discouraging scenario results from the high mutation rate occurring in HIV-1 envelope proteins and the complex structure of gp120 as an oligomer associated with gp41 (Kwong *et al.*, 1998; Wyatt *et al.*, 1998) that gives rise to numerous epitopes, with few of them accessible to neutralizing antibodies. Moreover, some epitope specificities may change during the course of disease as a result of viral evolution, antibody affinity maturation, and viral escape (Lewis *et al.*, 1998). Recent contributions have provided relevant insights into the crystal structure of the oligomeric HIV-1gp120 bound to CD4, as well as to a Fab antibody that is specific for the CCR5/CXCR4 virus-binding sites (Kwong *et al.*, 1998; Wyatt *et al.*, 1998). In further work, the unliganded SIV gp120 tertiary structure was reported (Chen *et al.*, 2005). Together, these structures explain the difficulty encountered in eliciting neutralizing antibodies. Main feature are summarized as follows: the CD4-binding site, a main neutralization-sensitive region is deeply hidden by the highly variable V1/V2 loops (Kwong *et al.*, 1998; Wyatt *et al.*, 1998). The CCR5-binding site, a neutralization-sensitive β-sheet structure, is absent in the unliganded structure and is functional only after CD4 binding and upon a large conformational change in the structure (shown in Fig. 5) (Chen *et al.*, 2005). Furthermore, the coreceptor-binding site is shielded by the hyper-variable V3 loop. Thus, the two potential neutralization-sensitive regions are well hidden in the oligomeric complex where most of the exposed surface is protected by sugars (Kwong *et al.*, 1998; Wyatt *et al.*, 1998).

Nevertheless, a small group of five neutralizing monoclonal antibodies specific for HIV envelope gp120 and gp41 have been made available (Binley *et al.* 2004; Calarese *et al.*, 2003; Cardoso *et al.*, 2005; Choe *et al.* 2003; Moulard *et al.*, 2002; shown in Table II). In particular, b12 binds to the CD4-binding site by using the rare feature of a long heavy chain

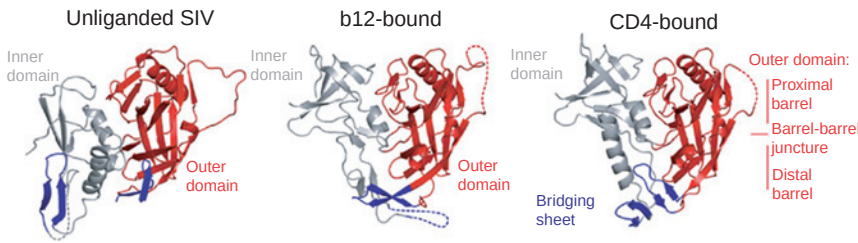


FIGURE 5 Conformational states of gp120. b12- and CD4-bound conformations of gp120 are depicted as in the unliganded conformation. Inner domains are gray, outer domains are red, with the disordered regions as dashed lines. Inner domains are depicted in red and the bridging sheet is shown in blue. Reprinted by permission from Macmillan Publishers Ltd.: *Nature*, Zhou *et al.*, 2007, © 2007 Nature Publishing Group.

TABLE II List of the Broadly Neutralizing Monoclonal Antibodies

Antibody	*Epitope specificity*	*Features*	*Reference*
b12	CD4-binding site	Extended CDR3 region	Saphire *et al.*, 2001
X5	CD4 induced (coreceptor-binding site)	Effective as Fab fragment only	Labrijn *et al.*, 2003
2G12	Complex mannose	Antibody has VH domain swap	Calarese *et al.*, 2003
2F5	Proximal domain of gp41	IgG3 with long CDR3; cross-reacts with cardiolipin	Haynes *et al.*, 2005; Zwick *et al.*, 2004
4E10	Conserved membrane	IgG3 with long CDR3; proximal domain of gp41 cross-reacts with cardiolipin	Cardoso *et al.*, 2005

complementary-determining region (CDR) 3 loop. A second antibody, X5, binds to the exposed CCR5/CXCR4-binding site only as a Fab fragment, suggesting that endogenous X5-like antibodies will not be as efficient as the Fab X5. A unique antibody, 2G12, recognizes a mannose determinant linked to the asparagines at positions 289, 332, and 396 (Scanlan *et al.*, 2002). The specific binding of 2G12 requires conformational changes in the heavy chain hinge region that cause a crossover of the variable regions and allow the generation of a large antigen-binding site (Calarese *et al.*, 2003).

Two broadly neutralizing human monoclonal antibodies, 2F5 and 4E10, bind to the membrane proximal region of gp41; the two linear adjacent epitopes are bound by the respective antibodies by long CDR3 regions. A structural analysis of 2F5 binding to the gp41 peptide revealed the unique feature of the hydrophobic tips of the heavy chain CDR3 region interacting with membrane lipid, thus extending the binding interactions (Ofek *et al.*, 2004). These characteristics are shared by antibodies specific for autoantigens, such as cardiolipin (Haynes *et al.*, 2005), and underscore the clinical relevance of the molecular mimicry in the generation of antibody response and raised some concerns on the feasibility of eliciting by vaccination an antibody response similar to 2F5 and 4A10 mAbs. (Haynes *et al.*, 2005).

1. Strategies for Eliciting Effective Antibody Responses

In the light of the disappointing results from envelope-based vaccine, the current approaches to elicit broadly neutralizing antibodies focus on strategies that may overcome the complexity and low immunogenicity of the oligomeric envelope. Several studies have attempted to increase the presentation of conformational epitopes by mild chemical inactivation of virus particles, or by using uncleaved oligomeric envelope into proteoliposome

preparations that are expected to mimic the envelope spikes of viral particles (Arthur *et al.,* 1998; Grundner *et al.*, 2002; Rossio *et al.*, 1998). These attempts, however, have failed to elicit an effective neutralizing antibody response (Grundner *et al.*, 2005; Lifson *et al.*, 2004; Poon *et al.*, 2005), likely as a consequence of low percentage of envelope proteins embedded into the lipid bilayer with the native conformation.

A similar approach has included the generation of virus-like particles (VLPs) (Doan *et al.*, 2005), which are expected to expose the envelope spike in a native-like conformation. In fact, VLPs may better mimic the structure of the surface envelope virions as compared with other types of antigen presentation. In addition, VLPs have been shown to stimulate both cellular and humoral responses (Doan *et al.*, 2005). This approach has been tested in several studies (Buonaguro *et al.*, 2002; Dale *et al.*, 2002; Montefiori *et al.*, 2001) where VLPs failed to elicit high levels of cross-neutralizing antibodies, a likely consequence of the inability of current VLP preparations to present functional forms of the spikes (Herrera *et al.*, 2005; Poignard *et al.*, 2003).

As discussed above, the accessibility of conserved antibody epitopes on the envelope is low and limits the effectiveness of a vaccine based on trimeric envelope glycoproteins. To overcome this hurdle, envelope spikes carrying truncated or modified variable regions have been tested (Barnett *et al.*, 2001; Lu *et al.*, 1998; Kang *et al.*, 2005; Kim *et al.*, 2003). Additional studies have focused on structural modifications of gp41 expected to improve the exposure of 2F5- and/or 4A10-like epitopes. However, these studies have resulted in small improvement in the neutralizing capacity of the immune sera (Barnett *et al.*, 2001) indicating that further studies are required to increase the immunogenicity of the envelope spike mimics.

Distinct approaches to overcome the HIV-1 genetic diversity have included multivalent antigen cocktails (Chakrabarti *et al.*, 2005; Cho *et al.*, 2001; Kim *et al.*, 2005; Lemiale *et al.*, 2001) and the use of proteins engineered according to a consensus of distinct clades or to ancestral HIV sequences selected by a computer-assisted analysis (Gao *et al.,* 2004; Gaschen *et al.*, 2002). The selected sequences are expected to elicit antibodies to multiple conserved regions of different viral clades (Gaschen *et al.*, 2002; Gao *et al.*, 2005). Results from these studies have shown little or no improvement of the breadth of the neutralizing antibody response (Mullins *et al.*, 2004). Indeed, these results indicate that a "universally shared" envelope protein that displays functional spikes may be very difficult or impossible to produce (Gao *et al.*, 2005; Mullins *et al.*, 2004). As shown in Fig. 5, some relevant features of the gp120 structure and the conformational changes it undergoes to bind its ligands have been revealed (Kwong, 2005; Zhou *et al.*, 2007). Gp120 and the linked gp41 are homotrimers. Thus, much of the surface of monomeric gp120 is hidden and not accessible to antibody (Wyatt *et al.*, 1998). The exposed surface is heavily glycosylated (Chen *et al.*, 2005); the carbohydrate moieties are added by host enzymes

and are poorly immunogenic in humans. The importance of sugars in protection against neutralization by antibodies was underscored by an experiment in which macaques were infected with SIV that had mutations designed to remove glycosylation sites around the V1/V2 loops. When macaques were infected with this virus, there was good initial control with high titers of neutralizing antibody; however, after a few weeks, virus escaped neutralization by adding the sugars by single-base substitutions (Reitter *et al.*, 1998).

2. *Mimotope Strategy to Induce Neutralizing Antibodies*

As mentioned eaerlier, a number of studies in animal models have demonstrated a protective role of antibodies against HIV-1 challenge (Burton, 1997; Chan and Kim, 1998; Eichberg *et al.*, 1992; Emini *et al.*, 1992; Gauduin *et al.*, 1997; Girard *et al.*, 1996; Mascola *et al.*, 1997; Zolla-Pazner *et al.*, 1998). However, these studies have failed to identify epitopes capable of inducing an effective neutralizing antibody response. In fact, the identity of the immunogenic epitopes has been determined in only a few mAbs and has remained elusive in the case of the human polyclonal immune response to multiple B-cell epitopes, which are primarily conformational in nature and cannot be identified from primary sequences (Boots *et al.*, 1997). In developing a protective vaccine, it would be advantageous to identify those epitopes that are specifically recognized by neutralizing antibodies generated by HIV-1-infected subjects. In fact, these epitopes would include a substantial proportion of the epitope repertoire generated among a large panel of HIV-infected subjects harboring different HIV-1 quasispecies. Moreover, these epitopes would function as antigenic mimics of HIV-1 and would induce antibodies reacting with the HIV-1 envelope when used as immunogens.

Combinatorial peptide libraries express a large collection of peptide sequences (10^8 or more) that mimic both linear and conformational epitopes of folded protein domains, and even carbohydrate structures (Cortese *et al.*, 1996) that contribute to the generation of viral escape variants (Reitter *et al.*, 1998). Such libraries might offer a unique opportunity to overcome the complexity of the HIV epitope repertoire by selecting a pool of HIV-specific mimotopes using HIV-specific antibodies generated in representative cohorts of HIV-infected subjects. In one study (Scala *et al.*, 1999), numerous epitopes were selected that behaved as antigenic and immunogenic mimics of HIV-1 or SHIV epitopes generated in the natural course of infection by HIV-infected subjects with good control of viremia. The selected epitopes functioned as structural mimotopes of distinct gp120 and gp41 epitopes, and induced a neutralizing antibody response when used as immunogens in mice. Of interest for vaccine formulation, the epitopes selected for reactivity with a given serum were recognized by antibodies from numerous subjects likely infected with different HIV-1 quasispecies (Scala *et al.*, 1999).

Epitopes, selected by using a clade B sera, were shared by a large percentage of sera from subjects from distant geographic regions infected with viral strains of clade A through F (Chen *et al.*, 2001), suggesting a cross-clade antigenicity. The above evidence underscores the capacity of phage-displayed peptide to mimic multiple HIV epitopes exposed *in vivo* on glycosylated gp120 without the constraint of a high sequence homology.

In further work, a pool of phage-displayed peptides (phagotopes) was tested for immunogenicity in the rhesus macaques SHIV model of infection, by using SHIV89.6PD as the challenge virus (Chen *et al.*, 2001). In this study, phagotopes-immunized animals showed lower levels of plasma peak viremia, protection from $CD4^+$ T cells depletion, and protection for AIDS-like disease (Chen *et al.*, 2001). In the light of the poor results from vaccine studies that rely on the development of complex (and expensive) oligomeric proteins that need to be processed by the immune system by a mostly uncontrolled mechanisms, the mimotope strategy may offer a large number of candidate antigenic epitopes to be tested as vaccine candidates.

IV. Concluding Remarks

The availability of a highly effective antiretroviral therapy has improved dramatically the clinical outcome of HIV-infected individuals; however, this health and societal advance is mostly restricted to individuals who live in the United States and Europe. Indeed, HIV infection continues to spread in underdeveloped Asian and African nations, with dramatic socioeconomic consequences. As a diffuse availability of drugs for an effective antiretroviral therapy is not in sight, an effective vaccine remains the most reliable solution. However, such a vaccine should afford a high rate of protection from primary infection to avoid ethical and economic constraints. Indeed, the large populations of HIV-infected or at at-risk subjects and their low income make the developing countries an attractive site to conduct extensive vaccine trials. However, a vaccine that protects a low percentage of enrolled individuals, as suggested by negative outcomes of gp120-based vaccine trials in the USA, will result in thousands of infected subjects that must be treated with the same expensive antiretroviral therapy available to HIV-infected subjects of developed countries. With the lack of efficient public health infrastructures, the economic and social burden of the expected vaccine breakthroughs will likely be very high. In this scenario, an effective vaccine would provide the best possibility of containing the AIDS pandemic. Indeed, although this review has discussed some of the ongoing strategies to devise an AIDS vaccine that induces T-cell immunity, the best hope for an effective vaccine will likely come from components that induce broadly neutralizing antibodies. In this regard, recent results have elucidated the complex crystal structures of trimeric envelopes under conditions of binding with CD4 or

with neutralizing antibodies. These studies have provided a better understanding of the mechanisms that preclude accessibility of the antibody response to neutralization-sensitive domains of the viral envelope. In parallel, crucial knowledge has come from the elucidation of the quaternary structure of neutralization epitopes complexed with the cognate neutralizing antibodies. Together, these studies will assist in designing a novel class of conformational epitopes capable of eliciting a cross-clade neutralizing antibody response.

References

Abel, K., Compton, L., Rourke, T., Montefiori, D., Lu, D., Rothaeusler, K., Fritts, L., Bost, K., and Miller, C. J. (2003). Simian-human immunodeficiency virus SHIV89.6-induced protection against intravaginal challenge with pathogenic SIVmac239 is independent of the route of immunization and is associated with a combination of cytotoxic T-lymphocyte and alpha interferon responses. *J. Virol.* **77**, 3099–3118.

Alter, G., Malenfant, J. M., Delabre, R. M., Burgett, N. C., Yu, X. G., Lichterfeld, M., Zaunders, J., and Altfeld, M. (2004). Increased natural killer cell activity in viremic HIV-1 infection. *J. Immunol.* **173**(8), 5305–5311.

Altfeld, M., Allen, T. M., Yu, X. G., Johnston, M. N., Agrawal, D., Korber, B. T., Montefiori, D. C., O'Connor, D. H., Davis, B. T., Lee, P. K., Maier, E. L., Goulder, P. J., *et al.* (2002). HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature* **420**, 434–439.

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., *et al.* (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69–74.

Amyes, E., Hatton, C., Montamat-Sicotte, D., Gudgeon, N., Rickinson, A. B., McMichael, A. J., and Callan, M. F. (2003). Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. *J. Exp. Med.* **198**(6), 903–911.

Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., Spiegel, H. M., Conlon, C., Spina, C. A., Havlir, D. V., Richman, D. D., *et al.* (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* **192**(1), 63–75.

Arthur, L. O., Bess, J. W., Jr., Chertova, E. N., Rossio, J. L., Esser, M. T., Benveniste, R. E., Henderson, L. E., and Lifson, J. D. (1998). Chemical inactivation of retroviral infectivity by targeting nucleocapsid protein zinc fingers: A candidate SIV vaccine. *AIDS Res. Hum. Retroviruses* **14**(Suppl. 3), S311–S319.

Baba, T. W., Jeong, Y. S., Pennick, D., Bronson, R., Greene, M. F., and Ruprecht, R. M. (1995). Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**, 1820–1825.

Baba, T. W., Liska, V., Khimani, A. H., Ray, N. B., Dailey, P. J., Penninck, D., Bronson, R., Greene, M. F., McClure, H. M., Martin, L. N., and Ruprecht, R. M. (1999). Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat. Med.* **5**, 194–203.

Barnett, S. W., Lu, S., Srivastava, I., Cherpelis, S., Gettie, A., Blanchard, J., Wang, S., Mboudjeka, I., Leung, L., Lian, Y., Fong, A., Buckner, C., *et al.* (2001). The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit

neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. *J. Virol.* **75**, 5526–5540.

Bhardwaj, N. (2001). Processing and presentation of antigens by dendritic cells: Implications for vaccines. *Trends Mol. Med.* 7(9), 388–394. (Review).

Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C. J., Burton, D. R., *et al.* (2004). Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J. Virol.* **78**, 13232–13252.

Birch, M. R., Learmont, J. C., Dyer, W. B., Deacon, N. J., Zaunders, J. J., Saksena, N., Cunningham, A. L., Mills, J., and Sullivan, J. S. (2001). An examination of signs of disease progression in survivors of the Sydney Blood Bank Cohort (SBBC). *J. Clin. Virol.* **22**, 263–270.

Boots, L. J., McKenna, P. M., Arnold, B. A., Keller, P. M., Gorny, M. K., Zolla-Pazner, S., Robinson, J. E., and Conley, A. J. (1997). Anti-human immunodeficiency virus type 1 human monoclonal antibodies that bind discontinuous epitopes in the viral glycoproteins can identify mimotopes from recombinant phage peptide display libraries. *AIDS Res. Hum. Retroviruses* **13**, 1549–1559.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M., and Oldstone, M. B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* **68**(9), 6103–6110.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B., and Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* **3**(2), 205–211.

Buonaguro, L., Racioppi, L., Tornesello, M. L., Arra, C., and Visciano, M. L. (2002). Induction of neutralizing antibodies and cytotoxic T lymphocytes in BALB/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. *Antiviral Res.* **54**, 189–201.

Burton, D. R. (1997). A vaccine for HIV type 1: The antibody perspective. *Proc. Natl Acad. Sci. USA* **94**, 10018–10023.

Calarese, D. A., Scanlan, C. N., Zwick, M. B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M. R., Stanfield, R. L., Roux, K. H., Kelly, J. W., Rudd, P. M., *et al.* (2003). Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science* **300**, 2065–2071.

Cardoso, R. M., Zwick, M. B., Stanfield, R. L., Kunert, R., Binley, J. M., Katinger, H., Burton, D. R., and Wilson, I. A. (2005). Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* **22**, 163–173.

Chakrabarti, B. K., Ling, X., Yang, Z. Y., Montefiori, D. C., Panet, A., Kong, W. P., Welcher, B., Louder, M. K., Mascola, J. R., and Nabel, G. J. (2005). Expanded breadth of virus neutralization after immunization with a multiclade envelope HIV vaccine candidate. *Vaccine* **23**, 3434–3445.

Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., *et al.* (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* **410**(6824), 106–111.

Chan, D. C., and Kim, P. S. (1998). HIV entry and its inhibition. *Cell* **93**, 681–684.

Chen, X., Scala, G., Quinto, I., Liu, W., Chun, T. W., Justement, J. S., Cohen, O. J., vanCott, T. C., Iwanicki, M., Lewis, M. G., Greenhouse, J., Barry, T., *et al.* (2001). Protection of rhesus macaques against disease progression from pathogenic SHIV-89.6PD by vaccination with phage-displayed HIV-1 epitopes. *Nat. Med.* **7**, 1225–1231.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., and Harrison, S. C. (2005). Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**, 834–841.

Cho, M. W., Kim, Y. B., Lee, M. K., Gupta, K. C., Ross, W., Plishka, R., Buckler-White, A., Igarashi, T., Theodore, T., Byrum, R., Kemp, C., Montefiori, D. C., *et al.* (2001). Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous simian/human immunodeficiency virus infection in pigtailed macaques. *J. Virol.* **75**, 2224–2234.

Choe, H., Li, W., Wright, P. L., Vasilieva, N., Venturi, M., Huang, C. C., Grundner, C., Dorfman, T., Zwick, M. B., Wang, L., Rosenberg, E. S., Kwong, P. D., *et al.* (2003). Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120. *Cell* **114**, 161–170.

Clerici, M., Levin, J. M., Kessler, H. A., Harris, A., Berzofsky, J. A., Landay, A. L., and Shearer, G. M. (1994). HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. *JAMA* **271**, 42–46.

Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., Cortese, I., Fortugno, P., Galfre, G., Nicosia, A., and Felici, F. (1996). Selection of biologically active peptides by phage display of random peptide libraries. *Curr. Opin. Biotechnol.* **7**, 616–621.

Curran, J. W., Morgan, W. M., Hardy, A. M., Jaffe, H. W., Darrow, W. W., and Dowdle, W. R. (1985). The epidemiology of AIDS: Current status and future prospects. *Science* **229**(4720), 1352–1357.

Dale, C. J., Liu, X. S., De Rose, R., Purcell, D. F., Anderson, J., Xu, Y., Leggatt, G. R., Frazer, I. H., and Kent, S. J. (2002). Chimeric human papilloma virus-simian/human immunodeficiency virus virus-like-particle vaccines: Immunogenicity and protective efficacy in macaques. *Virology* **301**, 176–187.

Deacon, J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C., Lawson, V. A., Crowe, S., *et al.* (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270**, 988–991.

Denesvre, C., Le Grand, R., Boissin-Cans, F., Chakrabarti, L., Hurtrel, B., Vaslin, B., Dormont, D., and Sonigo, P. (1995). Highly attenuated SIVmac142 is immunogenic but does not protect against SIVmac251 challenge. *AIDS Res. Hum. Retroviruses* **11**, 1397–1406.

Derdeyn, C. A., Decker, J. M., Bibollet-Ruche, F., Mokili, J. L., Muldoon, M., Denham, S. A., Heil, M. L., Kasolo, F., Musonda, R., Hahn, B. H., Shaw, G. M., Korber, B. T., *et al.* (2004). Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. *Science* **303**(5666), 2019–2022.

Doan, L. X., Li, M., Chen, C. Y., and Yao, Q. Z. (2005). Virus-like particles as HIV-1 vaccines. *Rev. Med. Virol.* **15**, 75–88.

Dorfman, T., Mammano, F., Haseltine, W. A., and Gottlinger, H. G. (1994). Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. *J. Virol.* **68**(3), 1689–1696.

Dorner, F., and Barrett, P. N. (1999). Vaccine technology: Looking to the future. *Ann. Med.* **31**, 51–60.

Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., *et al.* (2002). HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**(6884), 95–98.

Dyer, W. B., Ogg, G. S., Demoitie, M. A., Jin, X., Geczy, A. F., Rowland-Jones, S. L., McMichael, A. J., Nixon, D. F., and Sullivan, J. S. (1999). Strong human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte activity in Sydney Blood Bank Cohort patients infected with nef-defective HIV type 1. *J. Virol.* **73**, 436–443.

Edupuganti, S., Weber, D., and Poole, C. (2004). Cytotoxic T-lymphocyte responses to canarypox vector-based HIV vaccines in HIV-seronegative individuals: A meta-analysis of published studies. *HIV Clin. Trials* **5**, 259–268.

Eichberg, J. W., Murthy, K. K., Ward, R. H., and Prince, A. M. (1992). Prevention of HIV infection by passive immunization with HIVIG or CD4-IgG. *AIDS Res. Hum. Retroviruses* **8**, 1515.

Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoshi, S., Putney, S. D., Matsushita, S., Cobb, K. E., Jett, C. M., Eichberg, J. W., Murthy, K. K., *et al.* (1992). Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* **355**, 728–730.

Enose, Y., Kita, M., Yamamoto, T., Suzuki, H., Miyake, A., Horiuchi, R., Ibuki, K., Kaneyasu, K., Kuwata, T., Takahashi, E., Sakai, K., Shinohara, K., *et al.* (2004). Protective effects of nef-deleted SHIV or that having IFN-gamma against disease induced with a pathogenic virus early after vaccination. *Arch. Virol.* **149**, 1705–1720.

Evans, D. T., O'Connor, D. H., Jing, P., Dzuris, J. L., Sidney, J., da Silva, J., Allen, T. M., Horton, H., Venham, J. E., Rudersdorf, R. A., Vogel, T., Pauza, C. D., *et al.* (1999). Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat. Med.* **5**(11), 1270–1276.

Fonteneau, J. F., Larsson, M., Beignon, A. S., McKenna, K., Dasilva, I., Amara, A., Liu, Y. J., Lifson, J. D., Littman, D. R., and Bhardwaj, N. (2004). Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J. Virol.* **78**(10), 5223–5232.

Fultz, P. N., McClure, H. M., Swenson, R. B., McGrath, C. R., Brodie, A., Getchell, J. P., Jensen, F. C., Anderson, D. C., Broderson, J. R., and Francis, D. P. (1986). Persistent infection of chimpanzees with human T-lymphotropic virus type III/lymphadenopathy-associated virus: A potential model for acquired immunodeficiency syndrome. *J. Virol.* **58**(1), 116–124.

Gao, F., Korber, B. T., Weaver, E., Liao, H. X., Hahn, B. H., and Haynes, B. F. (2004). Centralized immunogens as a vaccine strategy to overcome HIV-1 diversity. *Expert Rev. Vaccines* **3**, S161–S168.

Gao, F., Weaver, E. A., Lu, Z. J., Li, Y. Y., Liao, H. X., Ma, B., Alam, S. M., Scearce, R. M., Sutherland, L. L., Yu, J.-S., Decker, J. M., Shaw, G. M., *et al.* (2005). Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group M consensus envelope glycoprotein. *J. Virol.* **79**, 1154–1163.

Gaschen, B., Taylor, J., Yusim, K., Foley, B., Gao, F., Lang, D., Novitsky, V., Haynes, B., Hahn, B. H., Bhattacharya, T., and Korber, B. (2002). Diversity considerations in HIV-1 vaccine selection. *Science* **296**, 2354–2360.

Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R., and Koup, R. A. (1997). Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. *Nat. Med.* **3**, 1389–1393.

Gibbs, J. S., Regier, D. A., and Desrosiers, R. C. (1994). Construction and *in vitro* properties of SIVmac mutants with deletions in "nonessential" genes. *AIDS Res. Hum. Retroviruses* **10**, 607–616.

Girard, M., Barre-Sinoussi, F., and van der Ryst, E. (1996). Vaccination of chimpanzees against HIV-1. *Antibiot. Chemother.* **48**, 121–124.

Goh, W. C., Markee, J., Akridge, R. E., Meldorf, M., Musey, L., Karchmer, T., Krone, M., Collier, A., Corey, L., Emerman, M., and McElrath, M. J. (1999). Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: Evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects. *J. Infect. Dis.* **179**, 548–557.

Gotte, M., Li, X., and Wainberg, M. A. (1999). HIV-1 reverse transcription: A brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Arch. Biochem. Biophys.* **365**(2), 199–210. (Review).

Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgana, B., Edwards, A., McMichael, A. J., Rowland-Jones, S., *et al.* (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* **3**, 212–217.

Grundner, C., Mirzabekov, T., Sodroski, J., and Wyatt, R. (2002). Solid-phase proteoliposomes containing human immunodeficiency virus envelope glycoproteins. *J. Virol.* **76**, 3511–3521.

Grundner, C., Li, Y., Louder, M., Mascola, J., Yang, X., Sodroski, J., and Wyatt, R. (2005). Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* **331**, 33–46.

Harro, C., Edupuganti, S., Goepfert, P., Priddya, F., Lally, M., Shiver, J., Li, X., Robertson, M., Leavitt, R., and Isaacs, R. (2005). Safety and immunogenicity of Adenovirus Type 5 (Ad5) HIV-1 gag vaccines *12th Conf. Retrovir. Oppor. Infect*, Abstract 504.

Haynes, B. F., Fleming, J., St. Clair, W. E., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R. M., Plonk, K., Staats, H. F., Ortel, T. L., Liao, H. X., *et al.* (2005). Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* **308**, 1906–1908.

Herrera, C., Klasse, P. J., Michael, E., Kake, S., Barnes, K., Kibler, C. W., and Moore, J. P. (2005). The impact of envelope glycoprotein cleavage on the antigenicity, infectivity, and neutralization sensitivity of Env-pseudotyped human immunodeficiency virus type 1 particles. *Virology* **338**, 154–172.

Hladik, F., Desbien, A., Lang, J., Wang, L., Ding, Y., Holte, S., Wilson, A., Xu, Y., Moerbe, M., Schmechel, S., and McElrath, M. J. (2003). Most highly exposed seronegative men lack HIV-1-specific, IFN-γ-secreting T cells. *J. Immunol.* **171**, 2671–2683.

Holmes, R. K., Koning, F. A., Bishop, K. N., and Malim, M. H. (2007). APOBEC3F can inhibit the accumulation of HIV-1 reverse transcription products in the absence of hypermutation. Comparisons with APOBEC3G. *J. Biol. Chem.* **282**(4), 2587–2595. Epub 2006 Nov 22.

Igarashi, T., Ami, Y., Yamamoto, H., Shibata, R., Kuwata, T., Mukai, R., Shinohara, K., Komatsu, T., Adachi, A., and Hayami, M. (1997). Protection of monkeys vaccinated with vpr- and/or nef-defective simian immunodeficiency virus strain mac/human immunodeficiency virus type 1 chimeric viruses: A potential candidate live-attenuated human AIDS vaccine. *J. Gen. Virol.* **78**, 985–989.

Iida, T., Kuwata, T., Ui, M., Suzuki, H., Miura, T., Ibuki, K., Takahashi, H., Yamamoto, T., Imanishi, J., Hayami, M., and Kita, M. (2004). Augmentation of antigen-specific cytokine responses in the early phase of vaccination with a live-attenuated simian/human immunodeficiency chimeric virus expressing IFN-gamma. *Arch. Virol.* **149**, 743–757.

Jekle, A., Schramm, B., Jayakumar, P., Trautner, V., Schols, D., De Clercq, E., Mills, J., Crowe, S. M., and Goldsmith, M. A. (2002). Coreceptor phenotype of natural human immunodeficiency virus with nef deleted evolves *in vivo*, leading to increased virulence. *J. Virol.* **76**, 6966–6973.

Johnson, R. P. (2002). Mechanisms of protection against simian immunodeficiency virus infection. *Vaccine* **20**, 1985–1987.

Johnson, R. P., and Desrosiers, R. C. (1998). Protective immunity induced by live attenuated simian immunodeficiency virus. *Curr. Opin. Immunol.* **10**, 436–443.

Johnson, R. P., Lifson, J. D., Czajak, S. C., Cole, K. S., Manson, K. H., Glickman, R., Yang, J., Montefiori, D. C., Montelaro, R., Wyand, M. S., and Desrosiers, R. C. (1999). Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: Inverse relationship of degree of protection with level of attenuation. *J. Virol.* **73**, 4952–4961.

Jones, T. (2002). Hepagene (PowderJect). *Curr. Opin. Investig. Drugs* **3**, 54–57.

Jones, N. A., Wei, X., Flower, D. R., Wong, M., Michor, F., Saag, M. S., Hahn, B. H., Nowak, M. A., Shaw, G. M., and Borrow, P. (2004). Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. *J. Exp. Med.* **200**(10), 1243–1256.

Kang, S. M., Quan, F. S., Huang, C. Z., Guo, L. H., Ye, L., Yang, C., and Compans, R. W. (2005). Modified HIV envelope proteins with enhanced binding to neutralizing monoclonal antibodies. *Virology* **331**, 20–32.

Kaul, R., Plummer, F. A., Kimani, J., Dong, T., Kiama, P., Rostron, T., Njagi, E., MacDonald, K. S., Bwayo, J. J., McMichael, A. J., and Rowland-Jones, S. L. (2000). HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J. Immunol.* **164**, 1602–1611.

Kaul, R., Rowland-Jones, S. L., Kimani, J., Dong, T., Yang, H. B., Kiama, P., Rostron, T., Njagi, E., Bwayo, J. J., MacDonald, K. S., McMichael, A. J., Plummer, F. A., *et al.* (2001). Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses. *J. Clin. Invest.* **107**, 341–349.

Kestler, H. V., 3rd, Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**, 651–662.

Kim, J. H., Pitisuttithum, P., Kamboonruang, C., Chuenchitra, T., Mascola, J., Frankel, S. S., DeSouza, M. S., Polonis, V., McLinden, R., Sambor, A., Brown, A. E., Phonrat, B., *et al.* (2003). Specific antibody responses to vaccination with bivalent CM235/SF2 gp120: Detection of homologous and heterologous neutralizing antibody to subtype E (CRF01.AE) HIV type 1. *AIDS Res. Hum. Retroviruses* **19**, 807–816.

Kim, Y. B., Han, D. P., Cao, C., and Cho, M. W. (2005). Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. *Virology* **305**, 124–137.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S., and Bhattacharya, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**, 1789–1796.

Kumar, A., Lifson, J. D., Silverstein, P. S., Jia, F., Sheffer, D., Li, Z., and Narayan, O. (2000). Evaluation of immune responses induced by HIV-1 gp120 in rhesus macaques: Effect of vaccination on challenge with pathogenic strains of homologous and heterologous simian human immunodeficiency viruses. *Virology* **274**, 149–164.

Kumar, A., Mukherjee, S., Shen, J., Buch, S., Li, Z., Adany, I., Liu, Z., Zhuge, W., Piatak, M., Jr., Lifson, J., McClure, H., and Narayan, O. (2002). Immunization of macaques with live simian human immunodeficiency virus (SHIV) vaccines conferred protection against AIDS induced by homologous and heterologous SHIVs and simian immunodeficiency virus. *Virology* **301**, 189–205.

Kuwata, T., Igarashi, T., Ido, E., Jin, M., Mizuno, A., Chen, J., and Hayami, M. (1995). Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their *in vitro* and *in vivo* replication. *J. Gen. Virol.* **76**(Pt 9), 2181–2191.

Kwong, P. D. (2005). Human immunodeficiency virus: Refolding the envelope. *Nature* **433**, 815–816.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.

Labrijn, A. F., Poignard, P., Raja, A., Zwick, M. B., Delgado, K., Franti, M., Binley, J., Vivona, V., Grundner, C., Huang, C.-C., Venturi, M., Petropoulos, C. J., *et al.* (2003). Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J. Virol.* **77**, 10557–10565.

Learmont, J., Tindall, B., Evans, L., Cunningham, A., Cunningham, P., Wells, J., Penny, R., Kaldor, A., and Cooper, D. (1992). Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* **340**, 863–867.

Lemiale, F., Brand, D., Lebigot, S., Verrier, B., Buzelay, L., Brunet, S., and Barin, F. (2001). Immunogenicity of recombinant envelope glycoproteins derived from T-cell line-adapted isolates or primary HIV isolates: A comparative study using multivalent vaccine approaches. *J. Acquir. Immune Defic. Syndr.* **26**, 413–422.

Lewis, J., Balfe, P., Arnold, C., Kaye, S., Tedder, R. S., and McKeating, J. A. (1998). Development of a neutralizing antibody response during acute primary human immunodeficiency virus type 1 infection and the emergence of antigenic variants. *J. Virol.* **72**, 8943–8951.

Lifson, J. D., Rossio, J. L., Piatak, M., Jr., Bess, J., Jr., Chertova, E., Schneider, D. K., Coalter, V. J., Poore, B., Kiser, R. F., Imming, R. J., Scarzello, A. J., Henderson, L. E., *et al.* (2004). Evaluation of the safety, immunogenicity, and protective efficacy of whole inactivated simian immunodeficiency virus (SIV) vaccines with conformationally and functionally intact envelope glycoproteins. *AIDS Res. Hum. Retroviruses* **20**, 772–787.

Louwagie, J., McCutchan, F. E., Peeters, M., Brennan, T. P., Sanders-Buell, E., Eddy, G. A., van der Groen, G., Fransen, K., Gershy-Damet, G. M., Deleys, R., *et al.* (1993). Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *AIDS* **7**(6), 769–780.

Lu, S., Wyatt, R., Richmond, J. F., Mustafa, F., Wang, S., Weng, J., Montefiori, D. C., Sodroski, J., and Robinson, H. L. (1998). Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res. Hum. Retroviruses* **14**, 151–155.

Luciw, P. A., Shaw, K. E., Shacklett, B. L., and Marthas, M. L. (1998). Importance of the intracytoplasmic domain of the simian immunodeficiency virus (SIV) envelope glycoprotein for pathogenesis. *Virology* **252**, 9–16.

MacGregor, R. R., Ginsberg, R., Ugen, K. E., Baine, Y., Kang, C. U., Tu, X. M., Higgins, T., Weiner, D. B., and Boyer, J. D. (2002). T-cell responses induced in normal volunteers immunized with a DNA-based vaccine containing HIV-1 env and rev. *AIDS* **16**, 2137–2143.

Mackay, G. A., Liu, Z., Singh, D. K., Smith, M. S., Mukherjee, S., Sheffer, D., Jia, F., Adany, I., Sun, K. H., Dhillon, S., Zhuge, W., and Narayan, O. (2004). Protection against late-onset AIDS in macaques prophylactically immunized with a live simian HIV vaccine was dependent on persistence of the vaccine virus. *J. Immunol.* **173**, 4100–4107.

Marthas, L., Sutjipto, S., Higgins, J., Lohman, B., Torten, J., Luciw, P. A., Marx, P. A., and Pedersen, N. C. (1990). Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *J. Virol.* **64**, 3694–3700.

Mascola, J. R., Louder, M. K., VanCott, T. C., Sapan, C. V., Lambert, J. S., Muenz, L. R., Bunow, B., Birx, D. L., and Robb, M. L. (1997). Potent and synergistic neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by hyperimmune anti-HIV immunoglobulin combined with monoclonal antibodies 2F5 and 2G12. *J. Virol.* **71**, 7198–7206.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L., and Lewis, M. G. (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* **6**, 207–210.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., and Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**(7037), 1093–1097.

McIntyre, L. B., Geczy, A. F., Dyer, W. B., Learmont, J. C., and Sullivan, J. S. (1999). The Sydney Blood Bank Cohort: A case-control study using a transfused HIV-1 seronegative group. *Ann. Epidemiol.* **9**, 436–440.

McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., *et al.* (2004). Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat. Med.* **10**, 1240–1244.

Miller, C. J., and Abel, K. (2005). Immune mechanisms associated with protection from vaginal SIV challenge in rhesus monkeys infected with virulence-attenuated SHIV 89.6. *J. Med. Primatol.* **34**, 271–281.

Montefiori, D. C., Baba, T. W., Li, A., Bilska, M., and Ruprecht, R. M. (1996). Neutralizing and infection-enhancing antibody responses do not correlate with the differential pathogenicity of SIVmac239delta3 in adult and infant rhesus monkeys. *J. Immunol.* **157**, 5528–5535.

Montefiori, D. C., Safrit, J. T., Lydy, S. L., Barry, A. P., Bilska, M., Vo, H. T., Klein, M., Tartaglia, J., Robinson, H. L., and Rovinski, B. (2001). Induction of neutralizing antibodies and gag-specific cellular immune responses to an R5 primary isolate of human immunodeficiency virus type 1 in rhesus macaques. *J. Virol.* **75**, 5879–5890.

Moulard, M., Phogat, S. K., Shu, Y., Labrijn, A. F., Xiao, X., Binley, J. M., Zhang, M.-Y., Sidorov, I. A., Broder, C. C., Robinson, J., Parren, P. W. H. I., Burton, D. R., *et al.* (2002). Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. *Proc. Natl. Acad. Sci. USA* **99**, 6913–6918.

Mullins, J. I., Nickle, D. C., Heath, L., Rodrigo, A. G., and Learn, G. H. (2004). Immunogen sequence: The fourth tier of AIDS vaccine design. *Expert Rev. Vaccines* **3**, S151–S159.

Myers, G. (1994). Tenth anniversary perspectives on AIDS. HIV: Between past and future. *AIDS Res. Hum. Retroviruses* **10**(11), 1317–1324. (Review; No abstract available).

Nilsson, C., Makitalo, B., Thorstensson, R., Norley, S., Binninger-Schinzel, D., Cranage, M., Rud, E., Biberfeld, G., and Putkonen, P. (1998). Live attenuated simian immunodeficiency virus (SIV)mac in macaques can induce protection against mucosal infection with SIVsm. *AIDS* **12**, 2261–2270.

Novembre, F. J., Saucier, M., Anderson, D. C., Klumpp, S. A., O'Neil, S. P., Brown, C. R., 2nd, Hart, C. E., Guenthner, P. C., Swenson, R. B., and McClure, H. M. (1997). Development of AIDS in a chimpanzee infected with human immunodeficiency virus type. *J. Virol.* **71**(5), 4086–4091.

Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R., and Kwong, P. D. (2004). Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. *J. Virol.* **78**, 10724–10737.

Parren, P. W., Marx, P. A., Hessell, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J. P., and Burton, D. R. (2001). Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization *in vitro*. *J. Virol.* **75**(17), 8340–8347.

Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C., and Picker, L. J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* **5**(5), 518–525.

Pitisuttithum, P., Berman, P. W., Phonrat, B., Suntharasamai, P., Raktham, S., Srisuwanvilai, L. O., Hirunras, K., Kitayaporn, D., Kaewkangwal, J., Migasena, S., Sheppard, H. W., Li, E., *et al.* (2004). Phase I/II study of a candidate vaccine designed against the B and E subtypes of HIV-1. *J. Acquir. Immune Defic. Syndr.* **37**, 1160–1165.

Poignard, P., Moulard, M., Golez, E., Vivona, V., Franti, M., Venturini, S., Wang, M., Parren, P. W., and Burton, D. R. (2003). Heterogeneity of envelope molecules expressed on

primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. *J. Virol.* **77**, 353–365.

Poon, B., Safrit, J. T., McClure, H., Kitchen, C., Hsu, J. F., Gudeman, V., Petropoulos, C., Wrin, T., Chen, I. S., and Grovit-Ferbas, K. (2005). Induction of humoral immune responses following vaccination with envelope-containing, formaldehyde-treated, thermally inactivated human immunodeficiency virus type 1. *J. Virol.* **79**, 4927–4935.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R., and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. USA* **94**(5), 1890–1895.

Pulendran, B., Palucka, K., and Banchereau, J. (2001). Sensing pathogens and tuning immune responses. *Science* **293**(5528), 253–256. (Review).

Quinto, I., Puca, A., Greenhouse, J., Silvera, P., Yalley-Ogunro, J., Lewis, M. G., Palmieri, C., Trimboli, F., Byrum, R., Adelsberger, J., Venzon, D., Chen, X., *et al.* (2004). High attenuation and immunogenicity of a simian immunodeficiency virus expressing a proteolysis-resistant inhibitor of NF-kappaB. *J. Biol. Chem.* **279**(3), 1720–1728.

Ratner, L., Fisher, A., Jagodzinski, L. L., Mitsuya, H., Liou, R. S., Gallo, R. C., and Wong-Staal, F. (1987). Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**(1), 57–69.

Reitter, J. N., Means, R. E., and Desrosiers, R. C. (1998). A role for carbohydrates in immune evasion in AIDS. *Nat. Med.* **4**, 679–684.

Rhodes, D., Solomon, A., Bolton, W., Wood, J., Sullivan, J., Learmont, J., and Deacon, N. (1999). Identification of a new recipient in the Sydney Blood Bank Cohort: A long-term HIV type 1-infected seroindeterminate individual. *AIDS Res. Hum. Retroviruses* **15**, 1433–1439.

Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Montefiori, D., Roberts, A., Buonocore, L., and Rose, J. K. (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* **106**, 539–549.

Rossio, J. L., Esser, M. T., Suryanarayana, K., Schneider, D. K., Bess, J. W., Jr., Vasquez, G. M., Wiltrout, T. A., Chertova, E., Grimes, M. K., Sattentau, Q., Arthur, L. O., Henderson, L. E., *et al.* (1998). Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *J. Virol.* **72**, 7992–8001.

Rowland-Jones, S. L., and McMichael, A. (1995). Immune responses in HIV-exposed seronegatives: Have they repelled the virus? *Curr. Opin. Immunol.* **7**(4), 448–455. Review.

Rowland-Jones, S. L., Dong, T., Fowke, K. R., Kimani, J., Krausa, P., Newell, H., Blanchard, T., Ariyoshi, K., Oyugi, J., Ngugi, E., Bwayo, J., MacDonald, K. S., *et al.* (1998). Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J. Clin. Invest.* **102**(9), 1758–1765.

Safrit, J. T., Andrews, C. A., Zhu, T., Ho, D. D., and Koup, R. A. (1994). Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: Recognition of autologous virus sequences within a conserved immunodominant epitope. *J. Exp. Med.* **179**(2), 463–472.

Sakuragi, S., Shibata, R., Mukai, R., Komatsu, T., Fukasawa, M., Sakai, H., Sakuragi, J.-I., Kawamura, M., Ibuki, K., Hayami, M., and Adachi, A. (1992). Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. *J. Gen. Virol.* **73**(Pt 11), 2983–2987.

Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R., and Wilson, I. A. (2001). Crystal structure of a neutralizing human IGG against HIV-1: A template for vaccine design. *Science* **293**, 1155–1159.

Scala, G., Chen, X., Liu, W., Telles, J. N., Cohen, O. J., Vaccarezza, M., Igarashi, T., and Fauci, A. S. (1999). Selection of HIV-specific immunogenic epitopes by screening random peptide libraries with HIV-1-positive sera. *J. Immunol.* **162**(10), 6155–6161.

Scanlan, C. N., Pantophlet, R., Wormald, M. R., Saphire, E. O., Stanfield, R., Wilson, I. A., Katinger, H., Dwek, R. A., Rudd, P. M., and Burton, D. R. (2002). The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of $\alpha 1 \geq 2$ mannose residues on the outer face of gp120. *J. Virol.* **76**, 7306–7321.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., *et al.* (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**(5403), 857–860.

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., and Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* **65**, 3514–3520.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z.-Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., *et al.* (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency -virus immunity. *Nature* **415**, 331–335.

Sumida, S. M., Truitt, D. M., Lemckert, A. A., Vogels, R., Custers, J. H., Addo, M. M., Lockman, S., Peter, T., Peyerl, F. W., Kishko, M. G., Jackson, S. S., Gorgone, D. A., *et al.* (2005). Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J. Immunol.* **174**, 7179–7185.

Ui, M., Kuwata, T., Igarashi, T., Ibuki, K., Miyazaki, Y., Kozyrev, I. L., Enose, Y., Shimada, T., Uesaka, H., Yamamoto, H., Miura, T., and Hayami, M. (1999a). Protection of macaques against a SHIV with a homologous HIV-1 Env and a pathogenic SHIV-89.6P with a heterologous Env by vaccination with multiple gene-deleted SHIVs. *Virology* **265**, 252–263.

Ui, M., Kuwata, T., Igarashi, T., Miyazaki, Y., Tamaru, K., Shimada, T., Nakamura, M., Uesaka, H., Yamamoto, H., and Hayami, M. (1999b). Protective immunity of gene-deleted SHIVs having an HIV-1 Env against challenge infection with a gene-intact SHIV. *J. Med. Primatol.* **28**, 242–248.

Van Baalen, C. A., Schutten, M., Huisman, R. C., Boers, P. H., Gruters, R. A., and Osterhaus, A. D. (1998). Kinetics of antiviral activity by human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL) and rapid selection of CTL escape virus *in vitro*. *J. Virol.* **72**(8), 6851–6857.

Veazey, R. S., Shattock, R. J., Pope, M., Kirijan, J. C., Jones, J., Hu, Q., Ketas, T., Marx, P. A., Klasse, P. J., Burton, D. R., and Moore, J. P. (2003). Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat. Med.* **9**, 343–346.

Vuola, J. M., Keating, S., Webster, D. P., Berthoud, T., Dunachie, S., Gilbert, S. C., and Hill, A. V. (2005). Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers. *J. Immunol.* **174**, 449–455.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985). Nucleotide sequence of the AIDS virus, *LAV Cell* **40**(1), 9–17.

Warren, J. (2002). Preclinical AIDS vaccine research: Survey of SIV, SHIV, and HIV challenge studies in vaccinated nonhuman primates. *J. Med. Primatol.* **31**, 237–256.

Whatmore, A. M., Cook, N., Hall, G. A., Sharpe, S., Rud, E. W., and Cranage, M. P. (1995). Repair and evolution of nef *in vivo* modulates simian immunodeficiency virus virulence. *J. Virol.* **69**, 5117–5123.

Willey, R. L., Byrum, R., Piatak, M., Kim, Y. B., Cho, M. W., Rossio, J. R., Jr., Bess, J., Jr., Igarashi, T., Endo, Y., Arthur, L. O., Lifson, J. D., and Martin, M. A. (2003). Control of viremia and prevention of simian-human immunodeficiency virus-induced disease in rhesus macaques immunized with recombinant vaccinia viruses plus inactivated simian

immunodeficiency virus and human immunodeficiency virus type 1 particles. *J. Virol.* **77**, 1163–1174.

Wilson, J. D., Ogg, G. S., Allen, R. L., Davis, C., Shaunak, S., Downie, J., Dyer, W., Workman, C., Sullivan, S., McMichael, A. J., and Rowland-Jones, S. L. (2000). Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* **14**(3), 225–233.

Wyand, M. S., Manson, K. H., Garcia-Moll, M., Montefiori, D., and Desrosiers, R. C. (1994). Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* **70**, 3724–3733.

Wyand, M. S., Manson, K., Montefiori, D. C., Lifson, J. D., Johnson, R. P., and Desrosiers, R. C. (1999). Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* **73**, 8356–8363.

Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., *et al.* (1998). The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705–711.

Yewdell, J. W., and Del Val, M. (2004). Immunodominance in TCD8 + responses to viruses: Cell biology, cellular immunology, and mathematical models. *Immunity* **21**, 149–153.

Yoshino, N., Ami, Y., Someya, K., Ando, S., Shinohara, K., Tashiro, F., Lu, Y., and Honda, M. (2000). Protective immune responses induced by a non-pathogenic simian/human immunodeficiency virus (SHIV) against a challenge of a pathogenic SHIV in monkeys. *Microbiol. Immunol.* **44**, 363–372.

Yu, X. G., Addo, M. M., Rosenberg, E. S., Rodriguez, W. R., Lee, P. K., *et al.* (2002). Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8 + T-cell responses following acute HIV-1 infection. *J. Virol.* **76**, 8690–8701.

Zaunders, J. J., Geczy, A. F., Dyer, W. B., McIntyre, L. B., Cooley, M. A., Ashton, L. J., Raynes-Greenow, C. H., Learmont, J., Cooper, D. A., and Sullivan, J. S. (1999). Effect of long-term infection with nef-defective attenuated HIV type 1 on CD4 + and CD8 + T lymphocytes: Increased CD45RO + CD4 + T lymphocytes and limited activation of CD8 + T lymphocytes. *AIDS Res. Hum. Retroviruses* **15**, 1519–1527.

Zhou, T., Xu, L., Dey, B., Hessell, A. J., Van Ryk, D., Xiang, S. H., Yang, X., Zhang, M. Y., Zwick, M. B., Arthos, J., Burton, D. R., Dimitrov, D. S., *et al.* (2007). Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature* **445**, 732–737.

Zhu, T., Corey, L., Hwangbo, Y., Lee, J. M., Learn, G. H., *et al.* (2003). Persistence of extraordinarily low levels of genetically homogeneous human immunodeficiency virus type 1 in exposed seronegative individuals. *J. Virol.* **77**, 6108–6116.

Zolla-Pazner, S., Lubeck, M., Xu, S., Burda, S., Natuk, R. J., Sinangil, F., Steimer, K., Gallo, R. C., Eichberg, J. W., Matthews, T., and Robert-Guroff, M. (1998). Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. *J. Virol.* **72**(2), 1052–1059.

Zwick, M. B., Komori, H. K., Stanfield, R. L., Church, S., Wang, M., *et al.* (2004). The long third complementarity-determining region of the heavy chain is important in the activity of the broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2F5. *J. Virol.* **78**, 3155–3161.

Nafees Ahmad

Department of Immunobiology, College of Medicine, University of Arizona,
Tucson, Arizona 85724

Molecular Mechanisms of HIV-1 Vertical Transmission and Pathogenesis in Infants

I. Chapter Overview

Human immunodeficiency virus type 1 (HIV-1) mother-to-infant transmission occurs at a rate of more than 30% and is the predominant cause of AIDS in children. Several maternal factors including advanced clinical stages, low $CD4^+$ lymphocyte counts, and high viral load, immune response, and disease progression have been implicated in an increased risk of vertical transmission. While use of antiretroviral therapy (ART) during pregnancy has been shown to reduce the risk of mother-to-infant transmission, selective

Advances in Pharmacology, Volume 56
1054-3589/08 $35.00

DOI: 10.1016/S1054-3589(07)56015-1

transmission of ART-resistant mutants has also been documented. More importantly, HIV-1-infected neonates and infants develop symptomatic AIDS more rapidly than infected adults, including their own infected mothers. By using HIV-1-infected mother–infant pairs as a transmitter–recipient model, the minor genotypes of HIV-1 with R5 phenotypes found in infected mothers were transmitted to their infants and initially maintained in the infants with the same properties. In addition, transmission of a major and multiple genotypes has been suggested. In the analysis of other regions of HIV-1 genome, we have shown a high conservation of intact and functional *gag* p17 and NC, *pol* RT, *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef* open reading frames (ORFs) following mother-to-infant transmission. Moreover, the HIV-1 sequences from nontransmitting mothers were less heterogeneous and accessory genes, *vif* and *vpr*, less functionally conserved compared with transmitting mothers and their infants. With respect to HIV-1 pathogenesis in infants, HIV-1 replicates more efficiently in neonatal mononuclear cells compared with adult cells, which is influenced at the level of HIV-1 gene expression. The increased HIV-1 gene expression and replication in neonatal target cells may contribute to a high viral load and rapid disease progression in infants. We should target the properties of transmitted viruses and those that are associated with disease progression for the development of effective, better, and new strategies for prevention and treatment of HIV-1 infection in infants.

II. Introduction

HIV-1 vertical (mother-to-infant) transmission is the major cause of AIDS in children, which has become one of the fastest growing aspects of the AIDS pandemic, as more women of childbearing age group are infected with HIV-1. These HIV-1-infected women are expected to give births to more than 300,000 HIV-1-infected infants every year worldwide. The United Nations Program on AIDS and the United States Agency for International Development project that a higher number of children will be infected with HIV-1 by the year 2010, with 90% of them in the developing countries. By the end of 2006, 39.5 million people were living with HIV/AIDS, including 17.7 million women and 2.3 million children. While 380,000 children died of AIDS, 530,000 were newly infected in 2006 worldwide (Global Summary of AIDS epidemic, 2006). In less developed countries, a significant proportion of all HIV-infected people are children, and it is estimated that one-third of all childhood deaths will be due to HIV-1 infection (Global Summary of AIDS epidemic, 2006). HIV-1 mother-to-infant transmission occurs at a rate of 30% and accounts for ~90% of all HIV-1 infections in children (Ahmad, 1996, 2000, 2005; Blanche *et al*., 1989; Hoff *et al*., 1988; Mok *et al*., 1987; Ryder *et al*., 1989; Scott *et al*., 1984; Sprecher *et al*., 1986; The European Collaborative Study, 1994; Weinbreck *et al*., 1988).

HIV-1-infected infants born to these infected mothers develop AIDS more rapidly than infected adults, including their own infected mothers. Also, the rate of HIV-1 vertical transmission depends upon the symptoms of the disease and the frequency of delivery at estimated rates of 24% in symptom-free mothers and 65% in mothers with disease or who have had a previous child with AIDS (Ahmad, 1996, 2000, 2005; Blanche *et al*., 1989; Hoff *et al*., 1988; Mok *et al*., 1987; Ryder *et al*., 1989; Scott *et al*., 1984; Sprecher *et al*., 1986; The European Collaborative Study, 1994; Weinbreck *et al*., 1988). However, the rate of HIV-2 transmission from mother to infant is much lower compared with HIV-1 (Adjorlolo-Johnson *et al*., 1994) and mothers infected with both HIV-1 and HIV-2 could transmit both viruses, but transmission of HIV-1 has generally been observed (Adjorlolo-Johnson *et al*., 1994).

HIV-1 vertical transmission occurs mainly at three stages: prepartum (transplacental passage), intrapartum (exposure of infants' skin and mucus membrane to maternal blood and vaginal secretions), and postpartum (breast milk) (Ahmad, 1996, 2000, 2005; Blanche *et al*., 1989; Hoff *et al*., 1988; Mok *et al*., 1987; Ryder *et al*., 1989; Scott *et al*., 1985; Sprecher *et al*., 1986; Weinbreck *et al*., 1988). Several maternal parameters including advanced clinical stages of the mother, low $CD4^+$ lymphocyte counts, maternal immune response to HIV-1, recent infection, high level of circulating HIV-1, and maternal disease progression have been implicated in an increased risk of mother-to-infant transmission of HIV-1 (Ahmad, 1996, 2000, 2005; Blanche *et al*., 1989; Hira *et al*., 1989; Hoff *et al*., 1988; Mok *et al*., 1987; Ryder *et al*., 1989; Scott *et al*., 1985; Sprecher *et al*., 1986; Weinbreck *et al*., 1988). Additional factors such as acute infection during pregnancy, the presence of other sexually transmitted diseases or other chronic infections, disruption of placental integrity secondary to chorioamnionitis and tobacco smoking have been shown to be associated with mother-to-infant transmission of HIV-1 (Report of a Consensus Workshop, 1992). While some studies have demonstrated a direct association between the presence of maternal antibody against the V3 domain of the envelope protein and a lower rate of transmission of HIV-1 (Devash *et al*., 1990; Rossi *et al*., 1989), whereas others have showed lack of correlation (Halsey *et al*., 1992; Parekh *et al*., 1991). The ability of maternal antibody to neutralize its own isolate (autologous neutralization) may be particularly important because it has been suggested that the virus mutants that are selected under immune pressure and cannot be neutralized may play a role in transmission (Scarlatti *et al*., 1993a). A recent study has examined the cloned envelope variants of 12 transmission pairs and found that vertically transmitted variants were resistant to neutralization by maternal plasma than were maternal viral variants near the time of transmission (Wu *et al*., 2006). Furthermore, these investigators found that the infant variants were the most neutralization resistant in the mother and had relatively few glycosylation sites in the envelope protein (Wu *et al*., 2006).

Progress has been made in understanding the molecular mechanisms of HIV-1 vertical transmission and pathogenesis in infected infants. This chapter will be dedicated in presenting data on the molecular and biological aspects of HIV-1 vertical transmission and molecular mechanisms of HIV-1 pathogenesis in infants.

III. Timing and Mechanism of HIV-1 Vertical Transmission

While the concrete mechanisms of HIV-1 vertical transmission are not known, the timing of HIV-1 transmission from mother to infant can occur mainly at prepartum (transplacental passage), intrapartum (exposure of infants skin and mucus membrane to maternal blood and vaginal secretions), and postpartum (breast milk). In an experimental animal model involving simian primates, simian immunodeficiency virus (SIV) has been found to be transmitted by all three routes including prepartum, intrapartum, and postpartum (McClure *et al.*, 1991). It has also been suggested that both cell-free and cell-associated virus can transmit infection, but their relative importance is unknown (Scarlatti *et al.*, 1993b). Examination of human placental and fetal tissue following termination of pregnancies provides substantial evidence that intrauterine infection of HIV-1 occurs (Douglas and King, 1992; Mulder-Kampinga *et al.*, 1993). Several studies have demonstrated the infection of placentas or fetuses by histological methods, polymerase chain reaction (PCR), or *in situ* hybridization (Lyman *et al.*, 1988) including the ability of certain placenta-derived cells to support HIV-1 replication *in vitro*. The capability of HIV-1 to pass through an intact placental barrier maintained *ex vivo* has been demonstrated (Bawdon *et al.*, 1994; Schwartz and Nahmias, 1991). HIV-1 antigens have also been found in the amniotic fluid (Calvelli and Rubinstein, 1990; Viscarello *et al.*, 1992) and may be related to the time of transmission. The zygote can be infected as early as the time of conception, probably by the virus present in vaginal secretions (Lairmore *et al.*, 1993), and then travels down the oviduct to the uterus, which is lined with macrophages that can also infect the dividing embryo on its passage to the uterus. The embryo may also be exposed to virus by uteral macrophages or residual seminal fluid (Lairmore *et al.*, 1993). These hypotheses were additionally supported by the presence of HIV-1 sequences and antigen in an 8-week fetus (Lairmore *et al.*, 1993).

Soon after the attachment of fetus to the uterus wall, the placenta is formed, by which most maternal–fetal exchanges take place (Scott, 1994). The presence of HIV-1 has been demonstrated in placental tissues (Maury *et al.*, 1989; Sprecher *et al.*, 1986). Since placental tissue is rich in monocytes and macrophages, the macrophage-tropic (MT) viral strain (R5 virus) can infect placental tissue and be transmitted to the fetus. The exact route by which HIV-1 crosses placenta is not known; however, the outer most layers of

the placenta, the terminal villi, and the trophoblast do posses CD4 receptor (Chandwani *et al*., 1991) that can be infected by HIV-1. The terminal villi and the trophoblasts are bathed in maternal blood and still all the fetuses are not infected by HIV-1. It is possible that HIV-1 replication is not supported in these cells (Chandwani *et al*., 1991) or a particular genotype or phenotype is needed to replicate in these cells. However, disruption of these cells could allow migration of the virus to the underlying cytotrophoblasts that may support HIV-1 replication and spread the virus via CD4 positive Hofbauer or endothelial cells to the fetal cells. HIV-1 can also be transmitted from mother to infant due to disruption of placental membrane as a result of viral, bacterial, and/or fungal infections, sexually transmitted diseases, and smoking (Nair *et al*., 1993; Scott, 1994). In addition, disruption of the placental membrane can occur as a result of chorioamnionitis (Scott, 1994). Chorioamnionitis has been shown to occur in ~20% of normal pregnancies (Scott, 1994) and that the percentage increases greatly in HIV-1-infected women (Nair *et al*., 1993). Moreover, it is likely that this would expose the underlying cells to maternal blood and increase the chance of transmission.

The intrapartum transmission (during or shortly before birth) occurs in more than 50% of the HIV-1 infection cases in infants (Bryson *et al*., 1992; Kourtis *et al*., 2006). Besides exposure to maternal blood at the time of labor and in the birth canal during delivery, HIV-1 has been found in cervical and vaginal secretions of infected women (Henin *et al*., 1993), suggesting that there may be an additional source of HIV-1 exposure for vaginally delivered infants. Moreover, if HIV-1 can be detected by virus culture or PCR in peripheral blood within 48 hours of birth, then the infection should be termed as intrauterine (Bryson *et al*., 1992; Henin *et al*., 1993). Some indirect evidence suggests that transmission may occur around the time of delivery. Several studies have demonstrated a lack of detectable virus in some infants at the time of birth, which turned positive after 3–6 months (Ehrnst *et al*., 1991; Krivine *et al*., 1992; Rouzioux *et al*., 1993). This could be attributed to relative insensitivity of testing at birth, a very small virus inoculum, or sequestration of the virus. Furthermore, studies of an animal model of perinatal transmission using SIV have shown that fetal monkeys can be infected during pregnancy (Davison-Fairburn *et al*., 1992; Fazely *et al*., 1993). In one study, placental disruption appeared to be an additional requirement for infection of fetus in monkeys (Davison-Fairburn *et al*., 1992). Moreover, the risk of transmission to a first-born twin has been found to be twofold higher than the second-born twin (Bulterys *et al*., 1992; Goedert *et al*., 1991) because the first child is exposed for a longer time to HIV-infected material in the birth canal than the second child. Further analysis of the European Collaborative Study (Rossi *et al*., 1989; The European Mode of Delivery Collaboration, 1999) has shown that the infection rate in children born by cesarean section was lower than those born via vaginal delivery.

Most of the postpartum HIV-1 vertical transmissions (Dunn *et al.*, 1992) occur via breast-feeding (Dunn *et al.*, 1992; Lepage *et al.*, 1987; Weinbreck *et al.*, 1988; Ziegler *et al.*, 1985), with estimated rates of 14% from mothers with established infection and 29% from mothers who acquire HIV-1 after birth (Dunn *et al.*, 1992). In developing countries, breast-feeding by HIV-1-infected mothers to their infants contribute significantly to HIV-1 transmission. HIV-1 transmission by breast milk may be related to the duration of exposure to breast milk, infectivity of the milk, specific susceptibility of the infant, or the timing of exposure (van't Wout *et al.*, 1994). HIV levels in breast milk correlate with viral load in plasma and most of the infections through breast-feeding occur within 3–6 months after birth, probably from colostrum or early milk (Nduati *et al.*, 2000; Rousseau *et al.*, 2004). The transmission of HIV-1 by breast-feeding is increased due to low maternal $CD4^+$ counts, mastitis, and prolong exposure (Nduati *et al.*, 1995; Semba *et al.*, 1999). The use of breast milk substitutes prevented 44% of infant infections and was associated with significantly improved HIV-1-free survival (Nduati *et al.*, 2000). While antibodies to HIV-1 in breast milk are not protective (Becquart *et al.*, 2000), early development of T-helper cell responses to HIV envelope showed protection. The purpose of further research should thus be to focus on the role of these immune responses in providing protection.

IV. Factors Associated with HIV-1 Vertical Transmission

Several viral and/or host factors protect a majority of children against HIV-1 infection born to HIV-1-infected mothers without any antiretroviral treatment (Connor and Ho, 1994; Hira *et al.*, 1989; Oleske *et al.*, 1983). Research has been focused in investigating these factors with the hope that they may provide information on new and important targets for perinatal interventions. Several maternal factors including advanced clinical stages of the mother, low $CD4^+$ lymphocyte counts, maternal immune response to HIV-1, recent infection, high level of circulating HIV-1, and maternal disease progression have been implicated in an increased risk of mother-to-infant transmission of HIV-1 (Adjorlolo-Johnson *et al.*, 1994; Ahmad, 1996, 2000, 2005; Blanche *et al.*, 1989; Hoff *et al.*, 1988; Mok *et al.*, 1987; Petropoulou *et al.*, 2006; Ryder *et al.*, 1989; Scott *et al.*, 1984; Sprecher *et al.*, 1986; The European Collaborative Study, 1994; Weinbreck *et al.*, 1988). In French Cohort study involving a 7-year follow-up, two factors were identified as being associated with an increased risk of maternal-to-fetal transmission: p24 antigenemia and elevated maternal age (Mayaux *et al.*, 1995). Furthermore, the risk of transmission increased gradually from 15% at counts of >600 $CD4^+$ cells to 43% at counts of <200 and was also related to the percentage of $CD8^+$ cells with the lowest risk (12%)

when the $CD4^+$ cell count was >500 and highest risk (50%) for cell count of <200 (Mayaux *et al*., 1995). Several studies indicate that elevated maternal viral load, plasma HIV-1 RNA levels, may play an important role in perinatal transmission (Garcia *et al*., 1999). Different threshold effects for transmission were observed, with 80% of the women transmitting with HIV-1 RNA levels over 100,000 copies/μl (Fang *et al*., 1995) and 75% of the women transmitting with HIV-1 RNA levels over 50,000 copies/μl (Dickover *et al*., 1996). However, several studies involving large size cohorts have been unable to significantly correlate a high viral load with increased risk of vertical transmission. Cao *et al*. (1997) and the investigators of the Ariel Project in the United States reported that the risk of transmission increased slightly with a higher viral load, but no threshold value of virus load was identified that discriminated between transmitters and nontransmitters. These investigators concluded that a high maternal viral load was insufficient to fully explain vertical transmission of HIV-1 (Cao *et al*., 1997). Several other studies reported similar results about no predictive threshold for maternal HIV-1 RNA was observed for vertical transmission (Mayaux *et al*., 1995).

Several other factors such as acute infection during pregnancy, the presence of other sexually transmitted diseases or other chronic infections, disruption of placental integrity secondary to chorioamnionitis, or smoking have been shown to be associated with mother-to-infant transmission of HIV-1 (Report of a Consensus Workshop, 1992). Since vitamin A deficiency and malnutrition can cause immunodeficiency and disruption of mucosal integrity and found to be associated with an increased risk of vertical transmission (Semba, 1997), vitamin A supplementation during pregnancy was found to be of no significant benefit (Burns *et al*., 1999). Several studies have demonstrated a direct association between the presence of maternal antibody against the V3 domain of the envelope protein and a lower rate of transmission of HIV-1 (Devash *et al*., 1990; Rossi *et al*., 1989), whereas others have showed lack of correlation (Halsey *et al*., 1992; Parekh *et al*., 1991). The ability of maternal antibody to neutralize its own isolate (autologous neutralization) may be particularly important because it has been suggested that the virus mutants that are selected under immune pressure and cannot be neutralized may play a role in transmission (Scarlatti *et al*., 1993a). Obstetrical factors such as mode of delivery, invasive monitoring, or duration of rupture membranes may alter the risk of intrapartum transmission (Douglas and King, 1992; Newell and Peckham, 1993; Report of a Consensus Workshop, 1992). The type of breast milk (colostrum vs later milk), duration of breast-feeding, and maternal factors such as viral load, antibody content of milk, and duration of mother's infection may also influence transmission (Report of a Consensus Workshop, 1992). In developed countries such as United States and others, breast-feeding is not recommended to HIV-1-infected mothers. In addition, the possibility of

viral factors affecting mother-to-infant transmission cannot be ruled out, since 70% of the children born to HIV-1-infected mothers are uninfected.

V. HIV-1 Infection Diagnosis in Neonates and Infants

Detection of HIV-1 antibodies in neonates and infants born to infected mothers is not immediately useful because maternal IgG HIV-1 antibodies cross placenta to the fetus and can persist for up to 18 months in the infant (Mok *et al*., 1987; Weinbreck *et al*., 1988). The infants born to HIV-1-infected mothers are evaluated at regular follow-ups up to 3 years before they are declared uninfected based on Center for Disease Control guidelines (Centers for Disease Control and Prevention, 1992). A specific immune response of the infant that would indicate infection is the presence of IgA or IgM antibodies, which do not cross the placenta (Nicholas *et al*., 1989), and can be used to diagnose HIV-1 infection in children born to seropositive mothers (Weiblen *et al*., 1990). In addition, HIV-1-infected children may be identified by measuring antibody production in the newborn's peripheral blood mononuclear cell (PBMC) cultures by using a B-cell mitogen (Amadori *et al*., 1988; Laure *et al*., 1988). However, the specific and reliable methods to detect HIV-1 in infants are PCR, antigen detection, virus culture, and *in vitro* antibody production. In a cohort study, the sensitivities of these tests were estimated and compared among each other and the sensitivity was found to be as follows: PCR (81.5%), virus culture (70.3%), *in vitro* antibody production (92.5%), and antigen (44.4%) (De Rossi *et al*., 1991). Usually, a positive PCR and virus culture in newborns are considered to be indicative of HIV-1 infection (Chouquet *et al*., 1997). Moreover, the PCR should be done on HIV-1 proviral DNA to detect the presence of HIV-1 in infected infants (Rogers *et al*., 1989).

VI. Prevention of HIV-1 Vertical Transmission

The AIDS Clinical Trials Group (ACTG) protocol 076 recommended that women with greater than 200 CD4 counts treated with zidovudine (ZDV), a nucleoside reverse transcriptase inhibitor (NRTI), during pregnancy can reduce the risk of HIV-1 vertical transmission in about two-third of the cases (Cooper *et al*., 1996). While oral ZDV was given to HIV-1-infected pregnant women between 14 and 34 weeks gestation and continued throughout pregnancy to target *in utero* transmission, ZDV was not administered during the first trimester because of the potential toxicity during the period of maximal organ development. ZDV was intravenously administered during labor, which rapidly crosses placenta and provides active level

of drug in the fetus during passage through the birth canal. Also, oral ZDV was given to the newborn for 6 weeks to inhibit viral replication in infant, if virus or infected maternal cells passed into the infant's circulation during uterine contraction. Furthermore, ACTG 185 perinatal trial that examined women with more advanced disease and lower CD4 counts than ACTG 076 (<200) showed a similar reduction in the transmission rate. The efficacy of ZDV in clinical practice has been evaluated and the results are consistent with the ACTG 076 and 185 trials. This treatment has become standard if the status of HIV-1 infection of pregnant women is known and has resulted in the reduction of HIV-1 transmission from mother to infant. Furthermore, a short course of ZDV (300 mg twice daily from 36 weeks gestation and every 3 hour from onset of labor till delivery) reduced perinatal transmission in Thailand (Shaffer *et al*., 1999) and Cote d'Ivoire (Wiktor *et al*., 1999). Based on the success of ACTG 076, the pediatric ACTG began new HIV prevention trials that build on the ZDV regimen. These trails included other antiviral agents and multidrug combination in an attempt to reduce mother-to-infant transmission even more than that achieved by ZDV alone. In early 1999, a study sponsored by The United Nations Program on AIDS of a combination regimen of ZDV and 3TC in three African countries showed promising results. The HIVNET 012 study proved that simple directly observed regimen of orally administered nevirapine, a nonnucleoside reverse transcriptase inhibitor (NNRTI), monotherapy (one dose to mother and one dose to the neonate) could produce significant reductions in the risk of vertical transmission in comparison with ZDV monotherapy in mothers who received no antepartum prophylaxis and who were permitted to breast-feed (Guay *et al*., 1999). Because NNRTI-based ART is used as a fine line of therapy in developing countries for prevention of HIV-1 vertical transmission, concerns have been raised as to whether single-dose nevirapine prophylaxis can compromise the efficacy of subsequent NNRTI-based ART regimens (Giaquinto *et al*., 2006). However, infected patients who have previously received a single-dose nevirapine as a part of mother-to-child transmission should be considered eligible for NNRTI-based regimens (Giaquinto *et al*., 2006). Furthermore, use of a single dose of nevirapine instead of ZDV was significantly more likely to decrease HIV-1 RNA in breast milk during the first week and through the third week postpartum and was associated with decreased transmission risk at six weeks (Chung *et al*., 2005). In addition, sustained breast milk HIV-1 suppression may contribute to the ability of nevirapine to decrease perinatal transmission of HIV-1 (Chung *et al*., 2005). Furthermore, highly active antiretroviral therapy (HAART) that includes three or more agents in combination of protease inhibitor or reverse transcriptase (RT) inhibitors has been used in small number of studies (McGowan *et al*., 1999) and low rates of vertical transmission were observed. Prospective studies using HAART are still in

progress. Perinatal transmission of ART-resistant mutant has been documented (Frenkel *et al*., 1995).

ZDV or other antivirals used during pregnancy may have long-term toxicities of the treatment, including adverse effects on mitochondrial dysfunction. One of the concerns is regarding intrauterine ZDV exposure, especially for large number of infants who will not be infected. The clinical manifestations of these mitochondrial toxicities include neuropathy, myopathy, cardiomyopathy, hepatic steatosis, lactic acidosis, and pancreatitis (Minkoff and Augenbraun, 1997). Moreover, the effect of ART in fetal and early life on total lymphocytes and CD8 cell counts was prolonged until at least eight years (Bunders *et al*., 2005). There are new findings of mitochondrial toxicity in infants who have been exposed to ART during pregnancy or delivery, but clinical implications are not clear (Moodley and Wennberg, 2005). The lethal effect of ZDV on embryonic development has been demonstrated in experimental animals. ZDV has been associated with noninvasive vaginal tumors in rats and mice after prolonged exposure to high doses and zalcitabine has inhibited normal lymphocyte function and caused thymic lymphomas and hydrocephalus in rodents (Minkoff and Augenbraun, 1997). However, the long-term toxicity is being evaluated in ACTG 219 protocol in infants up till age 21 years and protocol 288, which follows women from 076 for 3 years postpartum. The other concern is the development of ART resistance in women receiving ART during pregnancy and transmission of ART-resistant mutants to their infants. If we can differentiate transmitters from nontransmitters early in pregnancy by analyzing their viral genotypes in conjunction with maternal parameters (CD4 counts etc.), it will be helpful in making the decision as to which infected pregnant women should be placed on antiretroviral agents. This would then avoid adverse effects of the antivirals, if any, used during pregnancy and on the growth and development of uninfected children born to infected mothers.

VII. Characterization of HIV-1 Associated with Vertical Transmission

Better characterization of HIV-1 associated with vertical transmission may provide relevant information about the strategies of prevention and treatment because the strategies should be targeted at the properties of the transmitted viruses. In this context, several research groups have investigated the molecular and biological properties of HIV-1 that are transmitted from mothers to their infants. By comparing HIV-1 DNA sequences in the envelope V3 and V4–V5 regions from three mother–infant pairs, we and others have shown that a minor subtype of maternal virus from a genetically heterogeneous virus population was transmitted to the infant (Ahmad *et al*., 1995; Contag *et al*., 1997; Dickover *et al*., 2001;

Mulder-Kampinga *et al.*, 1995; Pasquier *et al.*, 1998; Sato *et al.*, 1999; Scarlatti *et al.*, 1993b; Wolinsky *et al.*, 1992). The minor HIV-1 genotype predominates initially as a homogeneous population in the infant and then becomes diverse as the infant grows older (Ahmad *et al.*, 1995). Several other groups have also reported transmission of a major (Dickover *et al.*, 2001; Scarlatti *et al.*, 1993b) or multiple (Dickover *et al.*, 2001; Lamers *et al.*, 1994; Pasquier *et al.*, 1998) HIV-1 genotypes from mother to infant. In a simian animal model involving SIV maternal-to-fetal transmission in five macaque pairs, selective transmission of single-SIV genotypes from mother to infant was revealed (Amedee *et al.*, 1995). In this transplacental transmission, the mothers harbored a heterogeneous virus population compared to their infants (Amedee *et al.*, 1995). Similar observations of selective transmission of HIV-1 have also been found in transmitter–recipient partners involving sexual transmission including a homogeneous sequence population present in the recipients (Cichutek *et al.*, 1992; McNearney *et al.*, 1990). Three models have been proposed to explain this feature: (1) the random dilution effect, in which a low inoculum of the virus is transmitted from the transmitter to the recipient; (2) selective amplification, in which multiple HIV-1 variants may enter the recipient but only one is selectively amplified; and (3) selective transmission, in which one viral variant has a selective advantage in penetrating the mucosal barrier of the new host (Zhu *et al.*, 1993). In mother-to-infant transmission, the selective transmission model looks the most favorable model as evidenced by the selective transmission of HIV-1 variants from mother to infant (Ahmad *et al.*, 1995; Contag *et al.*, 1997; Dickover *et al.*, 2001; Mulder-Kampinga *et al.*, 1995; Pasquier *et al.*, 1998; Sato *et al.*, 1999; Scarlatti *et al.*, 1993b; Wolinsky *et al.*, 1992). These findings are based only on the analysis of several regions in the *env* gene. My group has further characterized several other important regions in the HIV-1 genome associated with vertical transmission, as described in the following sections.

Several studies have also focused to characterize the biological properties of HIV-1 associated with horizontal and vertical transmission. The viral phenotype involved in sexual transmission has been shown to be uniformly MT and nonsyncytium inducing (NSI) in five HIV-1 seroconvertors including a homogeneous sequence population in the recipients (McNearney *et al.*, 1990; Zhu *et al.*, 1993). In addition, van't Wout *et al.* (1994) have shown that MT HIV-1 variants initiate infection after sexual, parenteral, and vertical transmission. In contrast, Kliks *et al.* (1994) in a small cohort study have demonstrated the transmission of a rapid or high-titered replicating T-cell tropic and neutralization-resistant HIV-1 variant from mother to child. Moreover, the viral phenotype in SIV transmission from mother to infant transplacentally was found to be MT (Amedee *et al.*, 1995).

My group took a molecular chimeric approach to characterize the biological properties of HIV-1 associated with mother-to-infant

transmission by evaluating the functional role of the V3 region from mother–infant isolates that we analyzed earlier (Lamers *et al*., 1994). Since the V3 region is the major determinant of replication efficiency, cell tropism, and cytopathic effects, reciprocally transferring the V3 regions into an HIV-1 infectious molecular clone would reveal the biological properties of HIV-1 involved in transmission (Ahmad *et al*., 2000; Rosenzweig *et al*., 1993). We found that all our V3 region chimeras were unable to replicate in T-lymphocyte cell lines but replicated in monocyte-derived macrophages (MDM) and primary blood lymphocytes (Matala *et al*., 2001). This data suggests that the V3 region from mother–infant isolates changed the tropism of the lymphotropic parent clone NL 4-3. We next examined the replication of these chimeras in HOS CD4-CCR5 and HOS CD4-CXCR4 cell lines that contain receptor (CD4) and coreceptors (CCR5 or CXCR4). Interestingly, the mother–infant V3 region chimeras infected and replicated in HOS CD4-CCR5 cell line but not HOS CD4-CXCR4 cell line. The data suggested that the V3 region from mothers and infants conferred macrophage tropism to the virus (R5 virus) (Matala *et al*., 2001). Furthermore, the syncytium-inducing ability of these chimeras were examined on MT-2 cells and found to be NSI or R5 (Matala *et al*., 2001). The characterization of the molecular and biological properties of HIV-1 variants transmitted from mother to infant will allow us to understand the molecular mechanisms of maternal transmission of HIV-1. These findings may aid in the development of strategies for the prevention and treatment of HIV-1 infection in children.

VIII. Chemokine Receptors and HIV-1 Vertical Transmission

Several chemokines receptors, including two distinct predominantly, CXCR4 and CCR5, referred as HIV coreceptors have been identified for the entry of T-lymphotropic (X4 virus) and MT (R5 virus) HIV-1, respectively (Alkhatib *et al*., 1996; Feng *et al*., 1996). The region responsible for determining coreceptor utilization was examined by Choe *et al*. (1996) and showed that V3 region of envelope gp120 was responsible for interacting with this coreceptor. The role of V3 region becomes very important in determining the tropism that may play an important role in transmission, infection, and disease progression. The R5 viruses are the more commonly transmitted viruses in sexual (Zhu *et al*., 1993) and vertical (Matala *et al*., 2001) transmissions. While R5 viruses predominate initially in most infected individuals, X4 viruses are more virulent and associated with a faster rate of $CD4^+$ T-cell loss following several years of infection (Connor and Ho, 1994; Richman and Bozzette, 1994). In addition, individuals homozygous for a 32-bp deletion in their CCR5 genes were substantially protected from HIV-1 infection and heterozygous for 32-bp deletion in CCR5 genes had a slow

disease progression (Dean *et al.*, 1996). Several other coreceptors that interact with HIV-1 have also been identified, including CCR2b, CCR3, CCR8, BOB, BONZO, and CX3CR1. Thus, the study of the viral genotypes and phenotypes controlled by the *env* gp120 and its interaction with the coreceptors (CXCR4, CCR5 etc.) may have significance for understanding viral transmission, pathogenesis, and disease progression. We and others have shown that R5 viruses are involved in maternal-to-fetal transmission.

The role of CCR5 on maternal-to-fetal transmission was investigated and it was found that infants who have two copies of 32-bp deletions in CCR5 were infectable by X4 viruses following vertical transmission. It was further suggested that that CCR5 deletion had a minimal to nonexistent effect on maternal-to-fetal transmission. However, in a study of 552 mother–infant pairs, no babies were found to be infected who were homozygous for 32-bp deletion for CCR5 (Philpott *et al.*, 1999). John *et al.* (2000) have shown that the maternal heterozygous SDF1 (a ligand for X4 HIV-1 coreceptor CXCR4) genotype (SDF1 3′A/wt) polymorphism was associated with increased risk of perinatal transmission of HIV-1 and particularly postnatal breast milk transmission. In contrast, the infant SDF1 genotype had no effect on mother-to-infant transmission (John *et al.*, 2000). Several additional studies should be performed to understand the role of chemokines receptors, especially CCR5, in HIV-1 vertical transmission.

IX. Molecular Properties of HIV-1 from Mother–Infant Pairs Associated with Vertical Transmission

My laboratory has been actively involved for over a decade in the analyses of various HIV-1 regions, including the structural genes (*gag, pol*, and *env*), regulatory (*tat* and *rev*), and accessory (*vif*, *vpr*, *vpu*, and *nef*) genes following perinatal transmission, with the idea that a complete molecular and biological characterization of HIV-1 associated with maternal-to-fetal transmission may provide relevant information for the strategies of prevention and treatment. These genes are essential for viral replication and pathogenesis and therefore should provide important information about the properties of the transmitted virus. We can then target the properties of HIV-1 that are involved in vertical transmission for preventive strategies.

The *env* gp120 binds to CD4 receptors and coreceptors located on the plasma membrane of $CD4^+$ T lymphocytes, monocytes, macrophages, and dendritic cell (Weiss, 1993). Comparison of the sequences of *env* genes from numerous HIV-1 isolates reveals a pattern of five variable regions (V1–V5) interspersed with conserved regions for the gp120 subunit. The potential pathogenic region of HIV-1 presumably resides within the *env* gene (Shioda *et al.*, 1991) and the variable regions. The hypervariable region 3, the V3 region, is functionally important in virus infectivity (Willey *et al.*, 1988),

association of gp120 with gp41 on the cell surface (Willey *et al*., 1988), virus neutralization (Matthews *et al*., 1986; Putney *et al*., 1986; Rusche *et al*., 1988), replication efficiency (Hwang *et al*., 1991; Shioda *et al*., 1991), and cellular tropism (Hwang *et al*., 1991; Shioda *et al*., 1991; Westervelt *et al*., 1992), whereas the V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread (Toohey *et al*., 1995). In addition, a functional interaction between V3 and the second conserved region is important for infectivity as well as syncytium formation and cell tropism (Koito *et al*., 1994; Stamatatos and Cheng-Mayer, 1993; Willey *et al*., 1994). Two distinct coreceptors, CXCR4 and CCR5, have been identified for the entry of T-lymphotropic and MT HIV-1, respectively (Alkhatib *et al*., 1996; Feng *et al*., 1996). The region responsible for interacting with the coreceptor was shown to be the V3 region (Choe *et al*., 1996). Thus, the role of V3 region becomes very important in determining the tropism that may play a key role in transmission, infection, and disease progression because MT HIV-1 (R5 viruses) are more commonly transmitted during sexual (Zhu *et al*., 1993) and vertical (Matala *et al*., 2001) transmissions. Thus, the study of the viral genotypes and phenotypes controlled by the *env* gp120 and its interaction with the coreceptors may provide important information about the molecular mechanisms of HIV-1 vertical transmission. HIV-1 also encodes *gag* p17 matrix protein that plays a pivotal role in the virus life cycle, including virus entry, localization to the nucleus, and virus assembly and release, and may have a role in transmission. In addition, the *gag* nucleocapsid (NC) plays a pivotal role in the viral life cycle, including encapsulating the viral genome, aiding in the reverse transcription process, protecting the viral genome from nuclease digestion, and packaging two copies of the viral genome into progeny virions (Feng *et al*., 1999; Freed, 1998; Heath *et al*., 2003; Krishnamoorthy *et al*., 2003; Poon *et al*., 1996; Zuber *et al*., 2000). The RT enzyme of HIV-1 also plays a crucial role in the life cycle of the virus by converting the single-stranded RNA genome into double-stranded DNA that integrates into the host chromosome (di Marzo Veronese *et al*., 1986; Gotte *et al*., 1998). In addition, the RT is also responsible for the generation of mutations throughout the viral genome including in its own sequences and is thus responsible for the generation of quasi-species in HIV-1-infected individuals.

The regulatory genes *tat* and *rev* and accessory genes *vif, vpr, vpu*, and *nef* are required for viral replication and pathogenesis. In addition, the accessory genes *vif* and *vpr* are found to be highly conserved and functional during natural infection (Goh *et al*., 1998; Sova *et al*., 1995), suggesting that *vif* and *vpr* are important for HIV-1 pathogenesis in maternal–fetal isolates. Since the transmitted viruses from mothers to infants are MT and NSI (R5 viruses), the role of HIV-1 accessory genes *vif* and *vpr* and p17 matrix becomes important because these proteins are necessary for HIV-1 replication in macrophages and provide relevant information toward the

development of strategies for prevention and treatment, because the strategies involved should be targeted at the properties of the transmitted viruses.

To determine the coding potential of the *env, gag* p17 and NC, *pol* RT, *env* (V3 region) and gp41, *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef* genes, we analyzed these sequences from five to seven infected mother–infant pairs following perinatal transmission (Ahmad *et al*., 1995; Hahn *et al*., 1999, 2003; Husain *et al*., 2001; Yedavalli *et al*., 1998a,b, 2001). The frequencies of the coding potential of the Gag p17, Gag NC, Pol RT, Env (V3 region) and gp41, Tat, Rev, Vif, Vpr, Vpu, and Nef ORFs were 86.2, 92.8, 87.2, 98.4, 84.17, 90.9, 93.9, 89.8, 92.17, 90.12, and 86.2%, respectively (Fig. 1). These data suggest that these ORFs are highly conserved following mother-to-infant transmission of HIV-1 and may have a role in perinatal transmission. The degree of variability of HIV-1 nucleotide and amino acid sequences including *gag* p17 and NC, *pol* RT, *env* V3 region and gp41, *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef* sequences (Ahmad *et al*., 1995; Hahn *et al*., 1999, 2003; Husain *et al*., 2001; Yedavalli *et al*., 1998a,b, 2001) is shown in Figs. 2 and 3, respectively. There was a low degree of sequence variability of HIV-1 sequences in the regions of *gag* p17 and NC, *pol* RT, *vif* and *vpr* compared to *env* V3 region, gp41, *tat*, *rev*, *vpu*, and *nef* sequences. In addition, most of

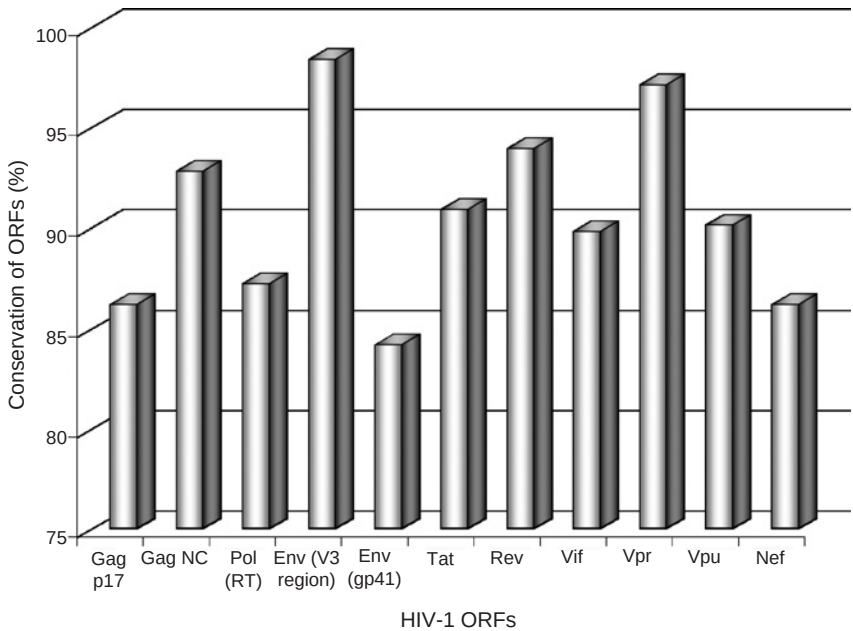


FIGURE 1 Frequency of conservation of intact HIV-1 ORFs, including Gag p17 and NC, Pol RT, Env (V3) and gp41, Tat, Rev, Vif, Vpr, Vpu, and Nef from deduced amino acid sequences from mother–infant isolates following vertical transmission. The frequency of conservation of ORFs is expressed as percentages of intact ORFs from mother–infant isolates.

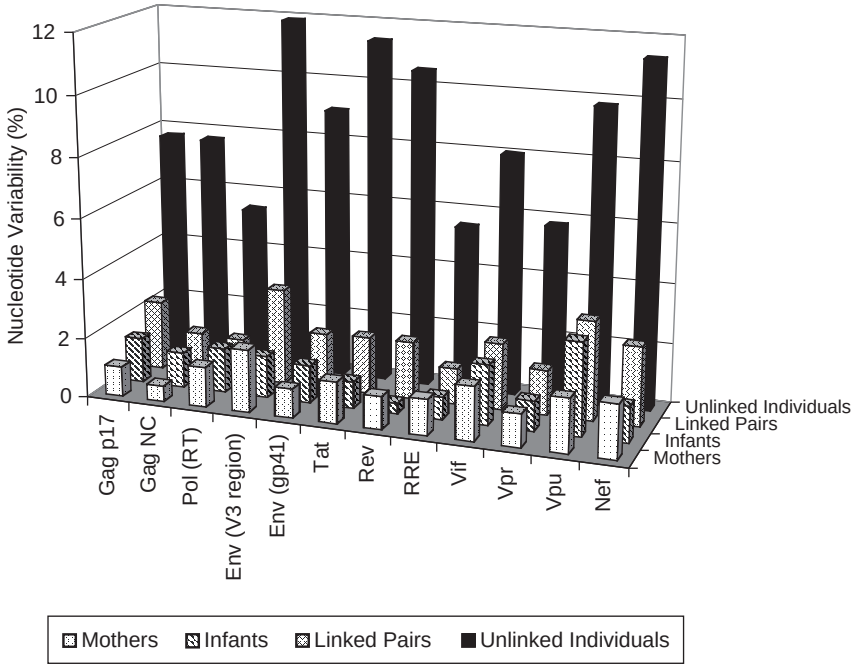


FIGURE 2 HIV-1 heterogeneity in the regions of *gag* p17 and NC, *pol* RT, *env* (V3 region) and gp41, *tat*, *rev*, RRE, *vif*, *vpr*, *vpu*, and *nef* nucleotide sequences from mother–infant isolates following vertical transmission. The percentages of mismatches were calculated between nucleotide sequences within same mother's set, within same infant's set, between epidemiologically linked mother–infant pair, and between epidemiologically unlinked individuals. The distance percentages were rounded off to the nearest decimal.

the mothers' HIV-1 sequences were more heterogeneous compared to infants' sequences, suggesting selective transmission. The genetic variability of HIV-1 nucleotide sequences was determined within mothers, within infants, and between epidemiologically linked and unlinked individuals as shown in Fig. 2. The data suggested that HIV-1 sequences from epidemiologically linked mother–infant pairs were closer than those from epidemiologically unlinked individuals. Interestingly, HIV-1 sequences in the conserved and less variable regions including *gag* p17 and NC, *pol* RT, *tat*, *vif*, and *vpr* were distinguishable from epidemiologically linked and unlinked individuals. Furthermore, the HIV-1 amino acid variability in the regions of Gag p17 and NC, Pol RT, Env, Tat, Rev, Vif, Vpr, Vpu, and Nef within mothers, within infants, and between linked mother–infant pairs is shown in Fig. 3. The phylogenetic analyses of HIV-1 genes, including *env* V3 region and gp41, *gag* p17and NC, *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef* sequences, from five to seven mother–infant pairs isolates following perinatal transmission were performed. The phylogenetic tree for 263

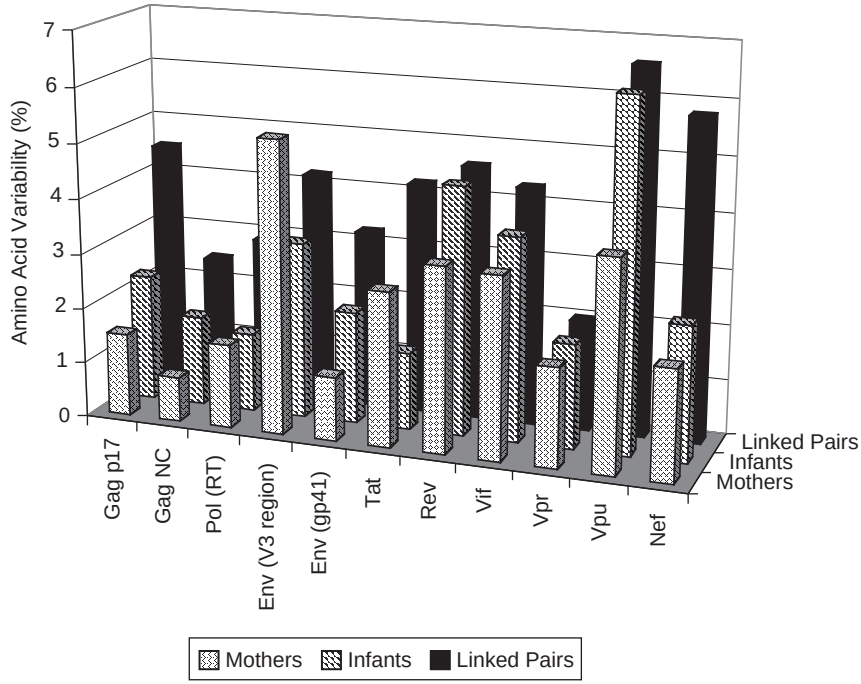


FIGURE 3 Genetic variability of HIV-1 Gag p17 and NC, Pol RT, Env (V3 region) and gp41, Tat, Rev, Vif, Vpr, Vpu, and Nef deduced amino acid sequences within mothers, infants, between epidemiologically linked mother–infant pairs. The percentages of mismatches were calculated between amino acid sequences within same mother's set, within same infant's set, and between epidemiologically linked mother–infant pair. The distance percentages were rounded off to the nearest decimal.

sequences for V3 region (Ahmad *et al*., 1995), gp41 (Ramakrishnan *et al*., 2006), 166 sequences for *gag* p17 (Hahn *et al*., 1999), 168 sequences for *gag* NC (Wellensiek *et al*., 2006), 132 sequences for RT (Sundaravaradan *et al*., 2005), 154 sequences for *tat* (Husain *et al*., 2001), 149 sequences for *rev* (Ramakrishnan *et al*., 2005), 137 sequences for *vif* (Yedavalli *et al*., 1998b), 166 sequences for *vpr* (Yedavalli *et al*., 1998a), 162 sequences for *vpu* (Yedavalli *et al*., 2001), and 196 *nef* (Hahn *et al*., 2003) sequences from five to seven mother–infant pairs revealed that all the mother–infant pairs were well discriminated, separated, and confined within subtrees (not shown), indicating that the epidemiologically linked mother–infant pairs were closer to each other and that there was no PCR product. We also performed a global phylogenetic tree for all our mother–infant pairs' sequences and other available HIV-1 sequences in these regions in the HIV databases and found that the mother–infant sequences were separated from other HIV-1 sequences. In addition, our mother–infant sequences grouped with the subtype or clade B.

X. Characterization of Functional Domains of HIV-1 Genes Associated with Vertical Transmission

The functional domains required for the activity of HIV-1 genes including Env V3 region and gp41, Gag p17and NC, Pol RT, Tat, Rev, Vif, Vpr, Vpu, and Nef were analyzed by comparing mother–infant pairs' deduced amino acid sequences with known HIV-1 strains. Since the amino acid sequence of V3 loop determines the tropism of HIV-1 (Hwang *et al*., 1991; Shioda *et al*., 1991), we compared our mother–infant V3 loop sequences with known lymphotropic and MT clones. Note that V3 loop sequences were closer to the MT clone than lymphotropic clone. The amino acids critical for tropism–tropism, including H at position 275, Y at 283, E or D at 287, T at 313 (Yang *et al*., 1996), were mostly present in all our mother–infant V3 loop sequences (Ahmad *et al*., 1995). Comparison of the motif SIHIGPGRALYTTGEIIGDI from position 274 to 291, that is generally conserved in MT clones and different in lymphotropic clones (Hwang *et al*., 1991; Shioda *et al*., 1991), with our mother–infant pair V3 region sequences (Ahmad *et al*., 1995) showed that the mother–infant V3 region sequences match best with the MT clone (Matala *et al*., 2001).

The functional domains and motifs required for gp41 activity in the deduced amino acid sequences including hydrophobic fusion peptide (FP), HR1, HR2, precursor (gp160) cleavage, and cell signaling were mostly conserved in our mother–infant pairs sequences. Mutations in the FLG motif of FP region that completely abolished syncytium-inducing ability and infectious virus production (Freed, 1998) were found to be highly conserved in most of our mother–infant pairs' sequences. In addition, none of our gp41 sequences harbored mutations in the leucine/isoleucine backbone of HR region, which have been shown to affect viral infectivity (Chan *et al*., 1997). Furthermore, no other mutations or substitutions were found in our mother–infant gp41 sequences that may affect (i) hydrophobic cavities in the HR1 region, (2) membrane fusion activity by destabilizing the trimer of hairpin structures, (3) packing interactions between the N- and C-terminal helices of gp41, and (4) the structural integrity of the trimer of hairpins (Lu *et al*., 2001).

HIV-1 gp41 typically contains three or four sites for N-glycan (N-X-S/T) attachment located at the C-terminus of the ectodomain, which may serve to modulate the exposure of HIV-1 proteins to immune surveillance in patients (Cheng-Mayer *et al*., 1999; Reitter *et al*., 1998). We found that there was relatively a high degree of conservation of the four glycosylation sites in the sequences analyzed with some changes in few sequences. Interestingly, a substitution of Ala613 → Thr was seen in our gp41 sequences (pair E) that has been previously shown to be derived from brain of AIDS patients (Ohagen *et al*., 2003). This suggests that the variants in patient E have the potential to be neurotropic and may cause central nervous system (CNS)

disorders, which are commonly seen in infected infants (Epstein and Sharer, 1994). In the other three glycosylation sites some changes were seen that do not affect the consensus motif. The membrane-spanning domain (MSD) that is critical for functional integrity (Miyauchi *et al.*, 2005) was highly conserved in our gp41 sequences, except in all sequences of mother G (Gly690 → Ser). As serine residues also have a stabilizing effect on the helix structure, it is possible that this substitution observed in mother G sequences might not compromise the functional activity. The endodomain encodes two lentivirus lytic peptides (LLPs) that inhibit Ca^{2+}-dependent T-cell activation (Tencza *et al.*, 1995) and a leucine zipper motif between LLP-1 and LLP-2 that plays an important role in HIV-1 replication and pathogenesis (Kao *et al.*, 2001). The gp41 sequences analyzed in our study showed a high degree of conservation of essential residues in this leucine zipper motif.

With respect to T20 target motifs, most of the clones analyzed in this study showed a high degree of conservation, which was expected because these patients were never treated with T20 inhibitors. In pair D sequences, four sequences in infant E (Asn554 → Ser) and two sequences in infant F (Asn554 → Gln) substitutions were found, suggesting that mutants naturally occurred in these patients and might be resistant to T20. A wide range of susceptibility to T20 has been described in FP naive virus isolates from patients (Labrosse *et al.*, 2003). Studies (Derdeyn *et al.*, 2000; Reeves *et al.*, 2002) have concluded that the susceptibility to T20 was influenced by coreceptor usage but not by polymorphisms in the gp41 N helix. These studies demonstrated that viruses containing a CCR5-utilizing V3 loop were four- to eightfold less susceptible to T20 than the CXCR4-utilizing parent strains. We and others have shown that CCR5-utilizing MT (R5) viruses are transmitted from mother to infant (Matala *et al.*, 2001; Wolinsky *et al.*, 1992), suggesting that T20 might help reduce viral load and may prevent perinatal transmission.

The Gag p17 matrix functional domains were examined in the deduced amino acid sequences of mother–infant isolates and found to be highly conserved. Several motifs in Gag p17 including glutamic acid (E) or aspartic acid (D) at position 55, tyrosine (Y) or phenylalanine (F) at position 79, an aspartic acid (D) or glutamic acid (E) at positions 93 and 102, and a dipeptide alanine-glutamic acids (AD) at positions 122–123 were present in most of the mother–infant pairs' sequences (Hahn *et al.*, 1999). Furthermore, the p17 motifs that were previously shown to be significantly associated with transmission (Narwa *et al.*, 1996), including a glutamic acid (E) at position 93, were found in one of seven mother–infant pairs and KIEEEQN at 103–109 in six of the seven mother–infant pairs. Taken together, these findings suggest that an intact and functional *gag* p17 ORF is essential for HIV-1 replication in mothers and infants and contains several motifs that may be associated with vertical transmission.

The functional domains in the NC and p6 genes from the six mother–infant pairs were mostly conserved during vertical transmission. The critical residues of the NC zinc fingers were highly conserved, while the basic residues throughout the NC protein displayed more variability. However, these changes within the basic residues did not result in an overall loss of these basic amino acids. In fact, most of the basic amino acids that were substituted were replaced by another basic residue. While some changes did result in a loss of a basic residue, other compensatory mutations elsewhere in the amino acid sequence replaced the residue lost. This conservation of critical zinc finger residues and presence of many basic residues imply that it is very important to the virus evolutionarily to maintain the function these motifs provide. The viral late domain, Vpr-binding sites, and AIP1-binding site were all mostly conserved as well, when the mother–infant p6 sequences were analyzed. Critical residues within the late domain, including the two prolines vital for Tsg101 binding, were highly conserved during vertical transmission. This domain was not only conserved but also duplicated within some mother D sequences. It has been shown that duplication of this domain could be linked to antiretroviral drug resistance (Ibe *et al.*, 2003a,b; Peters *et al.*, 2001). However, since mother D has not been exposed to antiretroviral drugs (Wellensiek *et al.*, 2006), this duplication must have arisen naturally or was present in the virus that was initially transmitted to mother D. It is not known at this point, however, what effect this duplication would have on the budding ability of the virus. Both of the Vpr-binding domains were mostly conserved in the mother–infant sequences obtained, as was the AIP1-binding motif. Again, the conservation of these functional domains during vertical transmission suggests that it is important to the virus evolutionarily to maintain the functions that these regions provide.

Examining the motifs of the deduced amino acid sequences of the RT gene from five mother–infant pairs, we found that the essential motifs required for RT activity were mostly conserved in our mother–infant pairs' sequences (Sundaravaradan *et al.*, 2005). The sites essential for primer binding, template binding, and positioning of template and primer, which are located in α-helix H and α-helix I (Jacobo-Molina *et al.*, 1993; Kohlstaedt *et al.*, 1992), were are all conserved in RT sequences (Sundaravaradan *et al.*, 2005). Specifically, the amino acids involved in recruitment of nucleotides during reverse transcription (Harris *et al.*, 1998) were mostly conserved. The active sites of the polymerase are located in the palm subdomain at the bottom of the DNA-binding cleft comprising of aspartic acid (D) residues at positions 110, 185, and 186 that were conserved within the five mother–infant pairs' RT sequences. Furthermore, the D185 and D186 also form a part of an essential YMDD motif, which is highly conserved in known HIV-1 isolates (Ding *et al.*, 1994, 1997; Huang *et al.*, 1998; Jacobo-Molina *et al.*, 1993; Kohlstaedt *et al.*, 1992; Mulky *et al.*, 2004) and also in our mother–infant pairs' RT sequences analyzed.

Some of the amino acids of the connexion subdomain that are critical for RNase H activity and replication (Gotte *et al*., 1999; Julias *et al*., 2002, 2003) are conserved in our RT sequences with several substitutions of compatible nature including V293I, K358R, A376S, and A390T. These substitutions were located in the regions of the connexion that forms the base of the binding cleft. It is possible that such mutations in the binding cleft may change the size of the cleft and affect fidelity of the RT without affecting the active site. Further assessment also shows that our RT sequences harbor mutations in the connexion and RNase H subdomains that are not at the critical sites required for RT activity. The implications of these mutations can be studied by performing the biological characterization of these RT clones in the context of HIV-1 replication. It would be interesting to determine the degree of genetic variability and conservation of RT functional domains in nontransmitting mothers and compare their sequences with the data presented here. Nonetheless, the data described here suggest that functional domains of the RT enzyme, including RT, DNA polymerase, and RNase H, were highly conserved in our five mother–infant pair sequences.

We analyzed the functional domains of Tat in our six mother–infant pairs' deduced amino acid sequences (Husain *et al*., 2001). The first coding exon of Tat consists of 72 amino acid and contains 5 functional regions or domains, which are responsible for all major known functions including binding of Tat to TAR region of nascent RNA (Rana and Jeang, 1999). The *tat* exon I contains several discrete regions that form two independent functional domains. These regions include the N-terminal acidic domain, rich in proline residues (amino acids 1–21); a cysteine-rich region (amino acids 22–37); a core region (amino acids 37–48) containing hydrophobic amino acids; and a basic TAR-RNA-binding region (amino acids 48–59) containing six arginines and two lysines (Wilson *et al*., 1999). The first functional domain, which consists of N-terminal, cysteine-rich, and core regions (amino acids 1–47), is an activation domain required for transactivation *in vivo* (Selby and Peterlin, 1990). The second functional domain contains the basic region and is required for both RNA binding (TAR binding) and nuclear localization activities of Tat (Selby and Peterlin, 1990). Examination of our *tat* sequences from six mother–infant pairs showed that all five discrete regions of *tat* exon I were conserved in most of the sequences from six mother–infant pairs (Husain *et al*., 2001). Each pair depicted a definite pattern of amino acid sequences and still preserved the basic structure of motifs in each discrete region, which make functional domains of Tat protein. An aspartic acid at position 61 was present across all the mother–infant sequences, whereas a glycine at position 42 was present in most of the pairs (Husain *et al*., 2001). In basic region that is involved in TAR-binding activity, a substitution was observed at position 59 where basic hydrophilic histidine was replaced either by another hydrophilic serine or by

a hydrophobic proline (Husain *et al*., 2001). The cysteine-rich region and core region, which form transactivation domain, showed high conservation with a few subtle changes (Husain *et al*., 2001). The hydrophilic asparagine at position 24 was present in some pairs and substituted by either proline, threonine, or lysine in other pairs (Husain *et al*., 2001). Similarly, hydrophobic isoleucine at position 39 was replaced by valine, methionine, leucine, or threonine in some pairs (Husain *et al*., 2001). We also compared all our mother–infant pairs' tat sequences with Table 1 of Jeang *et al*. (1999), and found that most of positions that contain substitutions are tolerable. However, the effect of the substitutions of lysine at position 29 and serine at 46 by arginine and tyrosine (Husain *et al*., 2001), respectively, on Tat activity is not clear.

Rev has two main functional domains. The N-terminal domain contains an arginine-rich sequence that serves both as the nuclear localization signal (NLS) (Cochrane *et al*., 1990) and as the RNA-binding domain (RBD) (Daly *et al*., 1989). This region is flanked by sequences that promote Rev multimerization (Thomas *et al*., 1998). The second essential domain within Rev is located near the C-terminus and has been mapped to residues 75–93, and is known to promote nuclear export signal (NES) (Malim *et al*., 1991). In addition, there are three leucine (L) residues at positions 78, 81, and 83 that are critical for Rev function and mutation of any of these leucines eliminates Rev activity (Malim *et al*., 1989). The Rev NLS is an arginine-rich sequence (35-RQARRNRRRRWRERQRQ-51). Four arginine (R) residues (at positions 35, 39, 40, and 44) participate in base-specific contacts with the high-affinity binding site in stems IIB and IID of RRE, whereas other residues, threonine (T) at position 34 and the R at 38, 41–43, 46, and 48, contact the sugar-phosphate backbone (Battiste *et al*., 1996). Single and double amino acid changes in the NLS from R38 → aspartic acid (D) and R39 → leucine (L) can prevent both nuclear import (Malim *et al*., 1989) and RNA binding (Hammerschmid *et al*., 1994). Also, replacement of the R at positions 35, 38, 39, or 44, the T at 34, or the asparagine (N) at 40 abolishes the *in vitro* RNA-binding potential of Rev (Olsen *et al*., 1991; Zapp *et al*., 1991). Examination of our six mother–infant pairs' *rev* sequences showed that the NLS/RBD were highly conserved (Fig. 2). Some substitutions including R44 → lysine (K), R45 → glutamic acid (E), and R46 → glycine (G) in one clone of mother B and R44 → K in one clone of infant D and mother F were observed.

We next examined the NES domain required for RNP complex formation involving Rev–RRE interaction necessary for mRNA export. Mutational analyses have demonstrated that disruption of the leucine-rich domain at positions 78, 81, and 83 yields dominant negative (M10) Rev proteins, which localize in the nucleus, bind with RRE-containing RNA, and multimerize, but fail to facilitate the export of these mRNAs from the nucleus to the cytoplasm (Daly *et al*., 1993). We found that these leucines were highly conserved in our mother–infant pairs' *rev* sequences.

Further, we analyzed the domains responsible for Rev multimerization and Rev–RRE interactions. Mutational analysis indicates that L18 in *rev* exon1 and isoleucine (I) 55 in *rev* exon 2 mediate Rev dimerization, whereas L12, valine (V) 16, and L60 form trimer assembly (Jain and Belasco, 2001). In addition, two regions of Rev (residues 12–22 and 52–60) join to create two composite protein surfaces for interaction with other Rev molecules. Mutations in any of the aliphatic residues including I at positions 19, L at 22, I at 52, or I at 59 reduce the affinity of Rev monomers to bind RRE stem-loop IIB, although these residues lie outside the RBD of Rev (Jain and Belasco, 2001). Furthermore, Raman spectroscopic studies suggest that the two regions of Rev implicated in multimerization are composed of a pair of α-helices, comprising of residues 9–24 and 34–62 (Watts *et al.*, 1998). The two α-helices contact each other over a limited region to form short antiparallel coiled coil with an intramolecular interface defined by I 19/L 22 and I 52/I 59. Thomas *et al.* (1998) have shown that multimerization-deficient mutants acted in a transdominant manner and that I 55 is the functionally important residue that produces a multimerization-deficient mutant (Thomas *et al.*, 1998). Examination of our mother–infant *rev* exon 1 and *rev* exon 2 sequences showed that the critical residues required for multimerization were mostly conserved, except I 55 was substituted by an L 55 in mother–infant pair C and by a phenylalanine (F) in pair F.

In the analysis of the functional domains of accessory genes, including Vif, Vpr, Vpu, and Nef, we found that functional domains in all these genes were conserved in most of the mother–infant pairs' sequences. In Vif, the two cysteines present at positions 114 and 133 (HXB2 clone numbering) are essential for viral infectivity and were present in most of the *vif* sequences at position 149. We also identified protein kinase C phosphorylation, N-myristoylation, cyclic AMP-dependent protein kinase phoshorylation, and cyclic GMP-dependent protein kinase phoshorylation sites present in *vif* sequences. Serine at position 144 is present in the motif SLQXLA (positions 144–149), which is the most highly conserved sequence among all lentiviruses Vif proteins (Oberste and Gonda, 1992), and mutation of serine to alanine at position 144 resulted in 90% inhibition of HIV-1 replication (Yang *et al.*, 1996). The SLQXLA motifs at positions 144–149 and the serine at position 144 were examined in *vif* amino acid sequences and found to be highly conserved in 135 of the 137 clones. The other important domain essential for membrane localization for Vif function during HIV-1 replication requires basic amino acids at the C-terminus such as lysine and arginine clustered at positions 157–160 and 173–184 were highly conserved, supporting the significance of these essential domains for Vif function during maternal–fetal transmission.

Three domains have been classified in the Vpr proteins (Mahalingam *et al.*, 1997) that are involved in virion incorporation, oligomerization, nuclear transport, cell cycle arrest, and differentiation. The first domain

includes amino acids 1–42 containing oligomerization domain and putative α-helix (amino acid residues 17–34) required for virion incorporation. The second domain encompasses a HS/FRIG motif related with cytoskeleton function (Macreadie *et al*., 1995), leucine/isoleucine-rich sequence (LR motif from amino acids 60–81) important for nuclear localization (Mahalingam *et al*., 1997), a conserved dipeptide (GC residues 75 and 76), and a potential α-helical motif (residues 46–74) postulated to play a role in virion incorporation and in the stability of Vpr (Mahalingam *et al*., 1997). The third domain (residues 77–96) contains highly charged amino acids and suggested to be involved in cell cycle arrest and differentiation (Mahalingam *et al*., 1995). The N-terminal domain of the Vpr contains five negatively charged amino acids that are highly conserved and predicts an amphipathic α-helix (Mahalingam *et al*., 1997). Examination of *vpr* sequences from six mother–infant pairs showed that the five negatively charged amino acids at the same positions were highly conserved (Yedavalli *et al*., 1998a). Based on mutational analysis, several studies (Mahalingam *et al*., 1995, 1997) have identified specific domains in Vpr required for virion incorporation, nuclear import, and cell cycle arrest/differentiation. The two glutamic acids at positions 21 and 24, hydrophobic polar leucines at 20, 22, 23, and 26, and alanine at 59 that are required for virion incorporation of Vpr (Mahalingam *et al*., 1997) were highly conserved in most of the 166 *vpr* sequences (Yedavalli *et al*., 1998a) analyzed. We then examined the nuclear transport properties of Vpr that comprises of glutamic acid at positions 21 and 24, leucine at 20, 22, 23, 26, 67, and 68, and alanine at 59 (Mahalingam *et al*., 1997) and found to be highly conserved in most of the mother–infant pairs' *vpr* sequences (Yedavalli *et al*., 1998a) analyzed. The cell cycle arrests properties of Vpr that require glutamic acid at positions 21 and 24, alanine at 30 and 59, leucine at 64 and 67, histidine at 71, glycine at 75, and cysteine at 76 (Di Marzio *et al*., 1995) as well as arginine at 73 and 80 (Mahalingam *et al*., 1995) were conserved in mother–infant pairs' *vpr* sequences (Yedavalli *et al*., 1998a).

While our understanding of Vpu function is incomplete, several studies have shown that Vpu can enhance the release of HIV-1 particles (Strebel *et al*., 1989; Terwilliger *et al*., 1989) as well as degrade CD4 molecule (Kimura *et al*., 1994) in the endoplasmic reticulum to allow the transport of HIV-1 Env protein to cell surface. Mutational analysis of Vpu has shown that N-terminal transmembrane domain of Vpu (amino acids 1–30) is critical for enhancing the release of viral particles from infected cells (Kimura *et al*., 1994; Strebel *et al*., 1989). The C-terminal domain, which has been predicted to contain two amphipathic α-helical domains located between amino acid residues 30–50 and 59–76 (Schubert *et al*., 1995), is required for binding to CD4 (Yao *et al*., 1995) and the phosphorylation of serines residues at positions 53 and 57 for degradation of CD4 (Schubert and Strebel, 1994; Tiganos *et al*., 1998). In addition, Tiganos *et al*. (1998) have

shown that a tryptophan at position 23 in the transmembrane domain (residues 6–28) was essential for CD4 binding and degradation. Comparison of *vpu* sequences from several HIV-1 isolates and subtypes identified amino acid residues highly conserved, including seven residues W23, Q36, E51, D52, G54, N55, and G59 (McCormick-Davis *et al.*, 2000), the initiating methionine, and the two conserved serines. These data suggested important roles for these residues for Vpu function in HIV-1 replication. Examination of our 162 mother–infant *vpu* sequences (Yedavalli *et al.*, 2001) revealed conservation of functional domains in most of the sequences with little variability among different clones and significant variability in infant D sequences in the region of first α-helical domain. The serine residues at positions 53 and 57 required for Vpu function (Bour *et al.*, 1995) were highly conserved in all of our Vpu sequences (Yedavalli *et al.*, 2001) analyzed. A tryptophan at position 23 required for CD4 binding and degradation (Schubert and Strebel, 1994) was also conserved in all *vpu* sequences (Yedavalli *et al.*, 2001). We also compared our mother–infant pair *vpu* sequences with those found in HIV databases (Myers *et al.*, 1995) and found similar conservation of functional domains (Yedavalli *et al.*, 2001).

Nef is an N-terminally myristoylated 206-amino acid protein ($\sim$27 kDa) (Ahmad and Venkatesan, 1988) and features a structurally flexible N-terminal arm of $\sim$70 residues and a conserved, highly structured core domain of $\sim$120 residues (residues $\sim$70–190). Since Nef acts as a connector between target molecules on the cell surface (CD4 and MHC-I) and the cellular protein-trafficking machinery, localization of Nef at the cell membrane is essential for its main functions and mutations in this region of Nef were shown to affect HIV-1 pathogenicity (Aldrovandi *et al.*, 1998). Furthermore, cell membrane association is required for adequate incorporation of Nef into maturing virions and may therefore be important for virion infectivity (Welker *et al.*, 1998). We found that the residues and motifs that form the membrane anchor arm (Welker *et al.*, 1998) were functionally highly conserved in all mother–infant sequences. Specifically, the myristoyl acceptor site (G^2) was highly conserved in all mother–infant sequences. The patch of basic amino acids (residues 4–22) was mostly conserved in the mother–infant sequences and the few observed substitutions do not grossly change the basic character of this region. The residues W^{57}, L^{58}, and E^{59} that comprise the direct CD4-binding site (Grzesiek *et al.*, 1996) were highly conserved in five of the seven mother–infant pairs (Hahn *et al.*, 2003). We also found that the dileucine-based endocytosis signal ($E/D^{160}xxxLL^{165}$) that can recruit APs to the cell membrane (Baur *et al.*, 1997) and, therefore, enhance internalization of CD4 as well as MHC-I was highly conserved in all our seven mother–infant pair sequences (Hahn *et al.*, 2003). In addition, the glutamic acid (E^{177}) was conserved in all mother–infant pairs' sequences, since the $E \rightarrow G^{177}$ mutation was shown to result in a dominant negative Nef protein that decreased HIV-1 production and infectivity (Fackler *et al.*, 2001).

Furthermore, the motifs important for MHC-I downregulation, the αH1 (Mangasarian *et al*., 1999), including the indispensable M^{20} and the flexible loop (residues 58–69) (Akari *et al*., 2000), were conserved in most of the mother–infant *nef* sequences (Hahn *et al*., 2003). Nef interacts with a multitude of host factors that are part of the cellular signaling machinery. The central point in these interactions is the proline-rich region P^{69}xxPxxPxxP^{78} that forms a left-handed polyproline type II helix (PPII) and allows Nef to directly bind to the SH3 domains of cellular kinases (Saksela *et al*., 1995), including Hck, Lyn, and Lck (Baur *et al*., 1997), and mutations in this region of the Nef selectively abolished the ability of Nef to block CD3 signaling (Iafrate *et al*., 1997). The four prolines of the P^{69}xxPxxPxxP^{78} motif were highly conserved in our mother–infant pairs' *nef* sequences (Hahn *et al*., 2003).

XI. Properties of HIV-1 Associated with Lack of Vertical Transmission

In addition to several host factors that influence HIV-1 vertical transmission, viral factors or determinants may also be involved in vertical transmission because more than 70% of the children born to HIV-1-infected mothers are uninfected in the absence of any antiretroviral therapy (Ahmad, 1996, 2000, 2005; Blanche *et al*., 1989; Hoff *et al*., 1988; Mok *et al*., 1987; Report of a Consensus Workshop, 1992; Ryder *et al*., 1989; Scott *et al*., 1984; Sprecher *et al*., 1986; The European Collaborative Study, 1994; Weinbreck *et al*., 1988). However, HIV-1 sequence analyses from nontransmitting mothers (mothers who failed to transmit the virus to their infants) are very limited. We have performed a systematic molecular analyses of HIV-1 isolates from nontransmitting mothers and compared with transmitting mothers' isolates with the idea that it may provide useful information about viral determinants associated with or lack of vertical transmission. Since the V3 region of HIV-1 envelope is an important component for virus neutralization, cellular tropism, infectivity, and replication, *gag* p17 matrix plays a pivotal role in HIV-1 life cycle, and accessory genes, *vif* and *vpr*, are conserved among most primate immunodeficiency viruses and confer selection for its function *in vivo*; viral determinants involved in the obviation of mother-to-infant transmission should be concentrated in this region. We, therefore, analyzed these regions from HIV-1-infected nontransmitting mothers and compared with previously analyzed sequences from transmitting mothers and their infants, as described below and shown in Fig. 4A.

The HIV-1 envelope V3 region sequences were analyzed from three nontransmitting mothers' (infected mothers who failed to transmit HIV-1 to their infants in the absence of antiretroviral therapy) PBMCs DNA

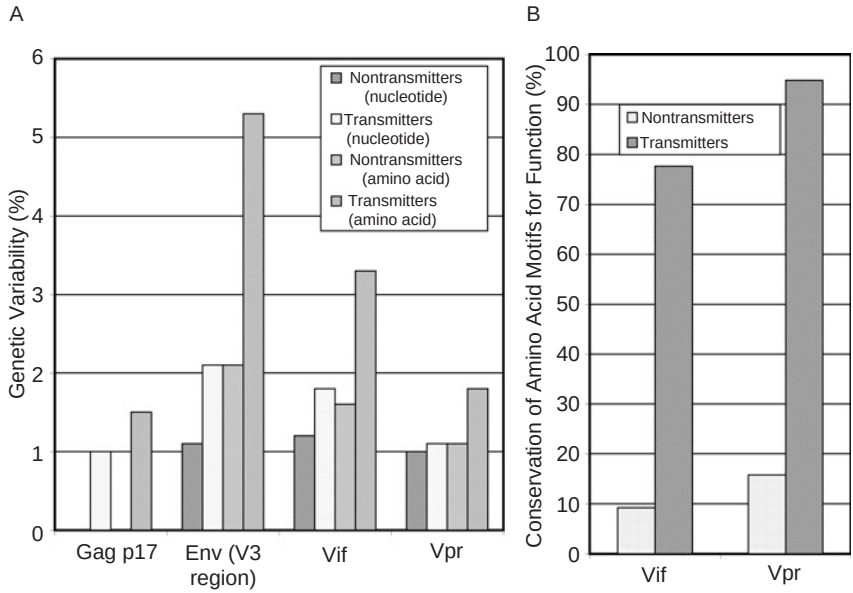


FIGURE 4 Comparison of (A) HIV-1 genetic variability (nucleotide and amino acid sequences) between transmitting mothers (infected mothers who transmitted HIV-1 to their infants) and nontransmitting mothers (infected mothers who failed to transmit the virus to their infants in the absence of ART) in the regions of *gag* p17, *env*-V3 region, *vif*, and *vpr* and (B) functional domains of Vif and Vpr required for HIV-1 replication in transmitting and nontransmitting mothers' isolates. The functional domains were determined based on previously described mutational analysis described in this chapter.

including one mother with two deliveries (Matala *et al*., 2000) and compared with our previously analyzed seven transmitting mothers' sequences (Ahmad *et al*., 1995). The coding potential of the envelope ORF as well as several patient-specific amino acid motifs and earlier described molecular features across the V3 region were highly conserved (Matala *et al*., 2000). There was a low degree of heterogeneity within each nontransmitting mother's sequence (Matala *et al*., 2000) compared to transmitting mothers' sequences (Ahmad *et al*., 1995). In addition, the estimates of genetic diversity of nontransmitting mothers' sequences were significantly lower compared to transmitting mothers' sequences (Matala *et al*., 2000). Phylogenetic analysis showed that each nontransmitting mother's sequences formed distinct clusters, which were well discriminated from each other and seven transmitting mothers' sequences (Matala *et al*., 2000). These data support the notion that a limited genetic diversity of HIV-1 in infected mothers may reduce the risk of maternal-to-fetal transmission (Matala *et al*., 2000), and this finding may be useful in developing strategies for further prevention of maternal-to-fetal transmission.

The *gag* p17 matrix sequences of HIV-1 were analyzed from three nontransmitting mothers (mothers who failed to transmit HIV-1 to their infants in the absence of ART), including multiple deliveries in case of one mother (Hahn and Ahmad, 2001). There was a low degree of heterogeneity of gag p17 matrix sequences in nontransmitting mothers (Hahn and Ahmad, 2001) compared to our previously analyzed mother–infant pairs' sequences. While most of the functional domains essential for p17 matrix function were generally conserved (Hahn and Ahmad, 2001), the polymerization site was less conserved. Several amino acid motifs including KIEEEQN (positions 103–109) at the major antibody-binding site was variable, and the C-terminal 6-mer QVSQNY, a lysine or glutamine at position 15, an alanine at 54, a lysine at 76, a valine at 104, and an aspartic acid at 102 and 121 were conserved in nontransmitting mothers' (Hahn and Ahmad, 2001) sequences compared to transmitting mothers' sequences (Hahn *et al.*, 1999). Phylogenetic analyses of 82 p17 matrix sequences revealed distinct clusters for each nontransmitting mother (Hahn and Ahmad, 2001). Some of these motifs in gag p17 matrix sequences that are present in nontransmitting mothers (Hahn and Ahmad, 2001) and absent in transmitting mothers (Hahn *et al.*, 1999) could be used as new targets for the development of preventive strategies for perinatal transmission.

The HIV-1 *vif* and *vpr* sequences were analyzed from four nontransmitting mothers (infected mothers who failed to transmit HIV-1 to their infants mainly in the absence of ART), including a mother with multiple deliveries (Yedavalli and Ahmad, 2001) and compared with the *vif* and *vpr* sequences of five and six previously analyzed transmitting mothers (Yedavalli *et al.*, 1998a,b), respectively. In contrast to a high functional conservation of *vif* and *vpr* genes in transmitting mothers' isolates (Yedavalli *et al.*, 1998a,b), we found that there was a low degree of conservation of functional domains of these genes in nontransmitting mothers' (NT) isolates (Yedavalli and Ahmad, 2001), as shown in Fig. 4B. For *vif* sequences, NT-2 contained stop codons and lack of initiation codons, whereas NT-1 sequences carried a substitution of a highly conserved tyrosine to histidine at position 30. In addition, NT-3 and NT-4 sequences contained additional substitutions, including aspargine at position 22, lysine at 77, and histidine at 110 that were absent in transmitting mothers' (Yedavalli and Ahmad, 2001) and consensus subtype B sequences. Similarly, the *vpr* sequences of NT-2 contained stop codons and lack of initiation codons, NT-4 a substitution of serine in place of alanine at position 30, some of NT-1 sequences arginine in place of glycine at position 75, and NT-3 sequences a deletion in the C-terminus (Yedavalli and Ahmad, 2001) that were absent in transmitting mothers' (Yedavalli *et al.*, 1998a,b) and consensus subtype B (Myers *et al.*, 1995) sequences and are essential for Vpr function. Furthermore, *vif* and *vpr* sequences of nontransmitting mothers (Yedavalli and Ahmad, 2001) were less heterogeneous compared to transmitting mothers' sequences

(Yedavalli *et al.*, 1998a,b). In conclusion, a low degree of conservation of functional domains and heterogeneity of HIV-1 *vif* and *vpr* genes in these infected mothers correlates with lack of vertical transmission (Yedavalli and Ahmad, 2001).

XII. Analysis of Immunologically Relevant Mutations in HIV-1 Isolates Associated with Vertical Transmission

Evasion of the host cytotoxic T-lymphocytes (CTL) response through mutation of key epitopes is a major challenge for both natural and vaccine-induced immune control of HIV-1. Immunodominant responses generally are effective but HIV-1 generates variants that expose CTL to a large pool of mutants impairing immune responsiveness (Rowland-Jones *et al.*, 1992). Escape from CTL control is indicated by a mutation that occurs in the T-cell epitope and becomes fixed in the virus population, resulting in an *in vivo* competitive advantage for the virus with reduction of the T-cell response to the wild-type epitope. Escape mutants can arise early or late in HIV-1 infection (Price *et al.*, 1997; Rowland-Jones *et al.*, 1992), and can also be transmitted (Goulder *et al.*, 2001). We have analyzed immunologically relevant mutations in several HIV-1 genes including Env gp41, Gag NC, Pol RT, and Rev associated with vertical transmission.

Geels *et al.* (2003) have described two epitope clusters of Env gp41 (residues 770–780 and 835–843) in HIV-1-infected patients over a period of 80 months that showed nonfixation of mutations. In the epitope encompassing residues 770–780, variant residues (Ile777 → Val and Val778 → Ile) were seen late (49 months) in infection, whereas in the other epitope (835–843), Ala836 → Thr and Tyr838 → Phe occurred early in infection. In the clones that were analyzed from five HIV-1-infected mother–infant pairs, Ile777 and Val778 were conserved across the board. However, several changes were observed, including a Cys838 → Leu (pair B), a Cys838 → Gly/Trp (pair D), a Cys838 → Phe (pair E), or Cys838 → Gly (pair F). These changes in the mother and infant clones suggest that these escape variants evolved to escape immune responses and influence transmission. In another study, infected mothers were found to transmit HIV-1 to their infants despite a strong CTL response to epitope 557–565 (RAIEAQQHL), suggesting generation of escape variants in the mothers (Wilson *et al.*, 1999). Mother–infant sequences in the current investigation showed a high conservation of this epitope with few substitutions in pair B (His564 → Arg) and D (Arg557 → Lys).

There have been several CTL epitopes identified within the NC and p6 proteins. The first epitope within the NC protein has the sequence CRAPRKKGC and is located between amino acid positions 28 and 36 (http://www.hiv.lanl.gov/content/immunology/ctl_search). This epitope is

recognized by HLA-B14 and contains the last cystine of the first zinc finger and the first cystine of the second zinc finger. Analysis of the NC amino acid sequences from the six mother–infant pairs revealed that this epitope was highly conserved in most of the clones that were obtained (Wellensiek *et al.*, 2006). Another CTL epitope, KEGHQMKDCTERQANF, is located at amino acid positions 42–57 and is recognized by several HLA types (http://www.hiv.lanl.gov/content/immunology/ctl_search). This epitope spans the last 14 amino acids of the NC protein and contains the histidine and final cystine of the second zinc finger. Again this epitope was mostly conserved when the sequences from the mother–infant pairs were analyzed. The next motif, CTERQANFL, is located from positions 50 to 56 and is recognized by HLA-B61 (http://www.hiv.lanl.gov/content/immunology/ctl_search). This epitope contains the last cystine of the second zinc finger and was highly conserved within the mother–infant sequences obtained.

The first motif within the p6 gene sequences, GNFLQSRPEPTAPPF, is located at amino acid positions 70–84 and is recognized by several HLA types (http://www.hiv.lanl.gov/content/immunology/ctl_search). Analysis of the mother–infant sequences revealed that this epitope was mostly conserved, with the exception of mother and infant C (pair C). Pair C contains a PTV insertion beginning at position 78 (Wellensiek *et al.*, 2006). It is not known at this time what effect on CTL recognition this insertion would have. The next epitope is located at amino acid positions 105–114 and has the sequence KELYPLTSL (http://www.hiv.lanl.gov/content/immunology/ctl_search). This epitope is recognized by HLA-B60 and is positioned within the $(LXX)_4$ Vpr-binding domain. Within the mother–infant sequences obtained this CTL epitope was mostly conserved; however, the first lysine within the epitope was substituted within every clone analyzed (Wellensiek *et al.*, 2006). It is not known at this time what effect this substitution would have on recognition of this epitope. Another epitope, YPLTSLRSLF, is located at positions 108–117 and is recognized by HLA-B7 (http://www.hiv.lanl.gov/content/immunology/ctl_search). Analysis of the six mother–infant pairs' sequences revealed that this epitope was mostly conserved (Wellensiek *et al.*, 2006). Overall, analyses of the CTL recognition epitopes within the sequences of the mother–infant pairs displayed that these epitopes, which are involved in immune recognition of the virus, were mostly conserved.

It has been shown that transmitting mothers have larger numbers of CTL escape variants as compared to nontransmitting mothers (Wilson *et al.*, 1999), emphasizing that CTL escape variants may become a part of circulating virus that influences vertical transmission (Menendez-Arias *et al.*, 1998; Wilson *et al.*, 1999). Several regions in the RT gene have been shown to elicit strong CTL responses during HIV-1 infection. The CTL eptitope, TVLDVGDAY, between amino acid positions 107 and 115 (http://www.hiv.lanl.gov/content/immunology/ctl_search) is highly conserved among

known HIV-1 isolates (Menendez-Arias *et al.*, 1998). This epitope contains the amino acid D110, which is a part of the RT active site. This epitope was highly conserved in most of the mother–infant RT clones sequenced (Sundaravaradan *et al.*, 2005). Another motif, TAFTIPSI, between amino acid positions 128 and 135 is an HLA-B51-restricted epitope (http://www.hiv.lanl.gov/content/immunology/ctl_search). This epitope is present in the palm region consisting of positions A129 and I135 as anchor residues (Menendez-Arias *et al.*, 1998). This motif was mostly conserved in the RT sequences of the five mother–infant pairs analyzed. In addition, I135T mutation decreases CTL response but increasing concentration of mutant peptide reestablishes appropriate responses (Menendez-Arias *et al.*, 1998). The I135T mutation was also seen in several of our mother–infant pair's D sequences.

The next motif AIFQSSMTK from amino acid positions 158–166, comprising of I159, F160, K166 anchor residues and recognized by several HLA types, is conserved among known HIV-1 isolates and believed to be associated with vertical transmission (Menendez-Arias *et al.*, 1998; Wilson *et al.*, 1999). Our mother–infant pairs' RT sequences showed conservation in this motif. Another CTL epitope YPGIKVRQL from positions 271 to 279 has been reported to be conserved in transmitting mothers and infants with several natural occurring variants (Wilson *et al.*, 1999), and was also found to be conserved in our mother–infant pairs' RT sequences. In addition, a P272H mutation that causes significant loss of CTL response for this epitope (Wilson *et al.*, 1999) was not seen in any of the RT clones analyzed.

Bobbitt *et al.* (2003) have demonstrated that when Rev activity was reduced by altering amino acids, especially L60 → F, HIV-1-infected cells were more resistant to anti-Gag and anti-Env CTL killing, suggesting that Rev influences CTL killing. Moreover, HIV-1-infected asymptomatic individuals were found to have a lower level of Rev activity, including lower levels of Gag and greater resistance to anti-Gag CTL killing (Bobbitt *et al.*, 2003). In the clones from the six mother–infant pairs analyzed, the sequences showed a high conservation of L 60, except in some clones from infant E (Ramakrishnan *et al.*, 2005). It has been shown that regions with high epitope motif density were less variable than regions with low epitope motif density (Kuiken *et al.*, 1999). In Rev, there are three high epitope motif dense regions (Korber *et al.*, 2003), namely RTVRLIKLLY (region 14–23 of *rev* exon 1, the multimerization domain), ISERILSTY (region 55–63 of *rev* exon 2, the multimerization domain), and SAEPVPLQLP (region 67–76, the NES). Interestingly, we observed variability within these three CTL epitope clusters, which were mostly patient specific. However, the CTL epitope ISERILSTY in the multimerization domain contained substitutions at positions E57G and R58 → Tryptophan (W) in most of sequences analyzed. The possibility exists that these changes give rise to CTL escape variants without compromising on Rev function but influencing transmission.

The defined CTL epitopes tend to cluster and for the Nef protein two immunodominant regions encompassing the amino acid positions 66–100 and 118–149 were identified (Price *et al*., 1997). Our results show that the proline-rich SH3-binding motif that is a part of the broadly recognized CTL epitope is highly conserved. However, a second CTL epitope that is recognized in most HLA backgrounds and includes, in addition to the SH3-binding motif, the downstream α-helix A displayed variability at positions 83–87 in five of the seven mother–infant pairs. The B8-restricted epitope (FLKEKGGL) at positions 90–97 that was described to initiate a strong CTL response early in infection (Wilson *et al*., 1999) was highly conserved in the mother–infant pairs' sequences. In the second epitope cluster, the epitope YFPDWQNYT, at positions 120–128, was described in a mother–infant setting (Wilson *et al*., 1999). This epitope may be found in mothers and infants with a Y to F variation at the first position, which did not change recognition. However, a Y to L variation that abrogated recognition was found only in infants (Price *et al*., 1997). Our results confirm that the Y to F variation is found in the mothers and infants. A study found Nef-specific CTL to be negatively correlated with age, with poor Nef-specific CD8 T-cell response in HIV-1-infected children compared with adults (Buseyne *et al*., 2006).

While perinatal transmission may be multifactorial in nature, characterization of HIV-1 transmitted from mother to infant may provide relevant information toward the development of strategies for prevention and treatment of HIV-1 infection in children. In this context, we (Ahmad *et al*., 1995) and others (Amedee *et al*., 1995; Contag *et al*., 1997; Dickover *et al*., 2001; Lamers *et al*., 1994; Mulder-Kampinga *et al*., 1995; Pasquier *et al*., 1998; Sato *et al*., 1999; Scarlatti *et al*., 1993b; Wolinsky *et al*., 1992) have shown a selective transmission of HIV-1 from mothers to their infants. Further molecular, biological, and immunologic characterizations described in this chapter may help AIDS researchers to target the specific variant of HIV-1 involved in transmission. We also show that the minor genotype transmitted from mother-to-infant is MT and NSI (R5 virus) (Matala *et al*., 2001). In addition, intact and functional *gag* p17 (Hahn *et al*., 1999), *vif* (Yedavalli *et al*., 1998b), *vpr* (Yedavalli *et al*., 1998a), *vpu* (Yedavalli *et al*., 2001), *nef* (Hahn *et al*., 2003), and *tat* (Husain *et al*., 2001) genes were conserved following mother-to-infant transmission. The conservation of intact and functional *vif* and *vpr* genes, which are essential for HIV-1 replication in macrophages, further supports our findings of MT and NSI phenotypes of the transmitted viruses (R5 viruses). More importantly, HIV-1 sequences from transmitting mothers were less heterogeneous than HIV-1 sequences from nontransmitting mothers (Hahn and Ahmad, 2001; Matala *et al*., 2000; Yedavalli and Ahmad, 2001). In addition, the accessory genes, *vif* and *vpr*, were less functionally conserved in nontransmitting mothers than transmitting mothers (Yedavalli and Ahmad, 2001). These results

might be helpful in understanding the pathogenesis of HIV-1 infection in mothers and infants including the molecular mechanisms involved in perinatal transmission, which may aid in the development of better strategies for prevention and treatment. In conclusion, we should target our preventive strategies on these molecular and biological properties of the virus.

XIII. Mechanisms of HIV-1 Pathogenesis and Disease Progression in Infants

HIV-1-infected infants have a higher level of viremia and develop AIDS more rapidly than infected adults, including their own infected mothers (Bamji *et al*., 1996). Moreover, the clinical manifestations in HIV-1-infected infants differ from those seen in their mothers and other infected adults (Calvelli and Rubinstein, 1990). Recurrent bacterial infections, lymphocyte interstitial pneumonitis, encephalopathy, neurological, and physical growth deficits are commonly observed in children with HIV-1 infection (Calvelli and Rubinstein, 1990). HIV-1-infected infants tend to have more CNS involvement than infected adults. This is further supported by the presence of HIV-1 in fetal CNS tissue (Lyman *et al*., 1988). The common opportunistic infection in both children and adults is *Pneumocystis carinii* pneumonia (Centers for Disease Control and Prevention, 1992). Several other common opportunistic infections in children include cytomegalovirus infections, Epstein-Barr virus (EBV) infections, herpes zoster, mycobacterium avium, cryptosporidium enteritis, chronic and recurrent mucosal and esophageal candidiasis, and mucocutaneous herpes simplex virus infection (van Dyke, 1993). A higher incidence of common childhood infections such as otitis media, sinusitis, viral respiratory infections, bacterial pneumonia, bacteremia, and meningitis have been observed in HIV-1-infected children (van Dyke, 1993). HIV-1-infected infants progress more rapidly from asymptomatic to symptomatic infection and from onset of symptomatic infection to death. The basis of more rapid progression to AIDS in infants is unknown. It is possible that HIV-1 interacts with the neonate's immune system in a different way than observed in adults. In neonates, HIV-1 replication is probably supported in the thymus. Thymocytes appear to differ from T cells in that they may support the replication of HIV-1 in the absence of any stimulatory effects (Schnittman *et al*., 1990). Thus, thymic injury from HIV-1 infection may have a profound impact on development of the immune system in HIV-1-infected fetuses and infants. Moreover, the thymus is critically important in infants and neonates in populating the immune system with T cells. The pediatric HIV-1 infection differs in immune dysfunction from those seen in adults. The immunologic abnormalities observed in HIV-1-infected infants and children include a decreased percentage of thymic $CD4^+$ cells, drastic reduction in cortical CD4/CD8 double

positive cells (Rubinstein *et al*., 1983; Stanley *et al*., 1993), and an increased percentage of $CD8^+$ cells (Koup and Wilson, 1993). In addition, the stromal cells, which support a thymocyte development, are damaged. In the periphery, an inverted ratio of CD4/CD8, an increased quantitative Ig, a decreased *in vitro* response to mitogens/antigens, a decreased CTL response, and a decreased phagocytosis have been reported (Koup and Wilson, 1993). In addition, poor antibody response to vaccination with T-dependent and T-independent antigens, increased production of IL-1β, IL-2, IL-6, and interferon-γ in lymph nodes, and decreased production of IL-2, IL-4, and interferon-γ by CD4 cells have been observed in HIV-1-infected children (Koup and Wilson, 1993).

The pathogenesis of HIV-1 infection in neonates and infants may be partially explained by relative immaturity of the immune system in early infancy, particularly in the neonatal period. Infants infected with HIV-1 have a more rapid and fatal course of HIV-1 disease than infected adults (Calvelli and Rubinstein, 1990). Most infants become symptomatic within the first few months of life; however, a subset of infants remains asymptomatic with laboratory evidence of immune abnormalities for years (Auger *et al*., 1988; Rogers *et al*., 1987). Since the infants' immune system is immature and developing, the immune responses generated against HIV-1 cannot contain the virus. Contrary to HIV-1-infected adults where strong CTL responses are associated with reductions in viremia, HIV-1-infected neonates generate HIV-1-specific $CD8^+$ T-cell responses early in life that are not clearly associated with reduction in viremia and improved clinical outcomes (Lohman *et al*., 2005). In contrast to X4 viruses associated with AIDS progression in adults (Cheng-Mayer *et al*., 1988; Koyanagi *et al*., 1987; Tersmette *et al*., 1988), rapidly progressing HIV-1-infected infants generally harbor viruses of R5 phenotype that is associated with high viral load (Cao *et al*., 1997; Hutto *et al*., 1996; Resino *et al*., 2000; Strunnikova *et al*., 1995). However, emergence of X4 variants (Resino *et al*., 2000; Strunnikova *et al*., 1995) in infected infants progressing to HIV disease and coreceptor change (Casper *et al*., 2002) has been reported. In terms of HIV-1 evolution and disease progression in infected children, Ganeshan *et al*. (1997) have shown a greater HIV-1 genetic distances relative to the time of infection in infected children with low virion-associated RNA and slow disease progression relative to those found in children with high virion-associated RNA and rapid disease progression. Zhang *et al*. (2006) have investigated the influence of viral genotypes and humoral immune response on disease progression in infected infants with subtype C, which has become the predominant subtype worldwide (Gordon *et al*., 2003; Jameel *et al*., 1995; Mandal *et al*., 2002; Oelrichs *et al*., 2000). These investigators found that rapid progressors received and maintained a genetically homogeneous viral population throughout the disease course, but slow progressors initially exhibited low levels of viral heterogeneity but attained higher levels of genetic diversity

over time (Zhang *et al*., 2006). However, there was no significant difference in the growth properties of viruses from rapid or slow progressors as well as humoral immune response was not predictive of disease progression (Zhang *et al*., 2006).

Different isolates of HIV-1 infect not only T lymphocytes but also other cells of the immune system, particularly monocytes and their mature form, macrophages. Infection of monocytes/macrophages (M/M) is of major importance because these cells are relatively refractory to the cytopathic effects of HIV-1 and may serve as a major reservoir for the virus as well as a vehicle for disseminating the virus to various tissues, including T cells and CNS. Much information related to the immunopathogenesis of AIDS has been gained from HIV-1 infection of primary adult MDM. The role of M/M, MDM, and lymphocytes in the immunopathogenesis of pediatric AIDS has not been fully determined. It is likely that the interaction of HIV-1 with the coreceptors for virus entry, enhancement of post-entry events for establishment of infection, and efficient virus replication due to cell activation in immature hosts' (neonates and infants) lymphocytes and MDM may be relevant to the enhanced susceptibility of neonates and infants to HIV-1 disease *in vivo*. Since R5 viruses have been shown to be involved in mother-to-infant transmission (Matala *et al*., 2001), interaction of R5 viruses with $CD4^+/CCR5^+$ positive T cells may also play an important role in pathogenesis of HIV disease. The majority of HIV-1-infected $CD4^+$ T cells in the blood of most infected infants and children have the memory ($CD45RO^+$) phenotype, despite the relative scarcity of these cells (Sleasman *et al*., 1996), because the $CD4^+$ T cells of infants are enriched in cells of the naive ($CD45RA^{high}$) phenotype that express higher levels of CXCR4 (Mo *et al*., 1998). Infection of naive ($CD45RA^+$) $CD4^+$ T cells is associated with a rapid decline in $CD4^+$ T-cell count in infants and children (Zaitseva *et al*., 1998) and may correlate with deaths as seen in adults (Ullum *et al*., 1997). As a primary site for fetal and neonatal production and selection of T cells, the fetal and neonatal thymus is the first site at which $CD4^+$ T cells are produced. It has been shown that thymus of HIV-infected children is atrophied (Joshi and Oleske, 1985), including severe depletion of lymphocytes and thymic epithelial cells (Grody *et al*., 1985) as well as depletion of T cells at a very early stage of differentiation (Rosenzweig *et al*., 1993). One way HIV-1 infection may decrease T-cell production is for the virus to cause a decrease in the number or activity of hematopoietic stem cells. We have shown that HIV-infected PBMCs have a diminished ability to produce $CD4^+$ and $CD8^+$ T cells in a thymic organ culture (Clark *et al*., 1997). Our preliminary results suggest that the development of $CD4^+$ cells from cord (immature) blood precursors is preferentially inhibited in HIV-1-infected human neonatal thymic organ cultures. On the other hand, because of the expansion of T-cell clones in the thymus, there is an expanding pool of newly generated $CD4^+$ cells that also expresses high levels of CXCR4, which

may favor amplification of X4 variants present in infants (Kitchen and Zack, 1997; Zaitseva *et al.*, 1998). The expansion of X4 variants would result in depletion of T-cell precursors (Balotta *et al.*, 1996). Since thymus is required for T-cell generation in infants, infection of thymocytes and thymic emigrants may have a major impact on disease progression in infants (Rosenzweig *et al.*, 1993). Therefore, elucidation of the mechanisms of HIV-1 infection in neonatal target cells may provide some insights into immunopathogenesis of HIV disease in infants.

We have compared HIV-1 replication kinetics between neonatal and adult blood mononuclear cells and determined the mechanisms of HIV-1 replication in these cell types. We have used cord blood in place of neonatal blood because, like neonatal blood, it has more $CD45RA^+$ T cells and less $CD45RO^+$ T cells, is immature compared with adult blood (Mo *et al.*, 1998), and is also available in a larger volume than neonatal blood. We have shown that HIV-1 replicates more efficiently in cord blood MDM and T lymphocytes compared with adult blood cells (Sundaravaradan *et al.*, 2006). There was no significant difference in the cell proliferative capabilities, in the levels of HIV-1 receptor (CD4) and coreceptors (CXCR4 and CCR5) for virus entry, and in the levels of post-entry events [reverse transcription and translocation of preintegration complex (PIC) into the nucleus] of cord blood mononuclear cells versus adult mononuclear cells (CBMCs vs PBMCs) (Sundaravaradan *et al.*, 2006). However, there was a significant upregulation in HIV-1 gene expression in cord MDM and T lymphocytes compared with adult cells, suggesting that the differential HIV-1 replication in cord and adult target cells is regulated at the level of HIV-1 gene expression (Sundaravaradan *et al.*, 2006).

To determine the replication efficiency of HIV-1 in cord and adult target cells, we infected cord and adult blood T lymphocytes (1×10^6) and MDM (0.5×10^6) from seven different donors with HIV-1. Equal number of lymphocytes and MDM isolated were infected with equal amount ($5–15 \times 10^4$ RT counts per minute) of $HIV\text{-}1_{BaL}$ and $HIV\text{-}1_{NL4\text{-}3}$ (laboratory adapted R5 and X4 HIV-1, respectively). Culture supernatants were replaced every 3 days and assayed for virus production by measuring RT activity (Ahmad and Venkatesan, 1988; Ahmad *et al.*, 1989; Matala *et al.*, 2001). The results of $HIV\text{-}1_{BaL}$ replication in cord and adult blood T lymphocytes and MDM are shown (Sundaravaradan *et al.*, 2006). The data clearly demonstrates that $HIV\text{-}1_{BaL}$ replicated threefold better in cord T lymphocytes compared with adult T lymphocytes at peak RT production (Fig. 5A). Moreover, this effect was more profound in MDM, where $HIV\text{-}1_{Ba\text{-}L}$ replicated ninefold better in cord MDM compared with adult MDM (Fig. 5B). We also found that $HIV\text{-}1_{NL4\text{-}3}$ replicated threefold better in cord T lymphocytes compared with adult T lymphocytes (Sundaravaradan *et al.*, 2006). These data demonstrate that there was an increased replication efficiency of HIV-1 in both cord T lymphocytes and MDM compared with adult cells, with a more

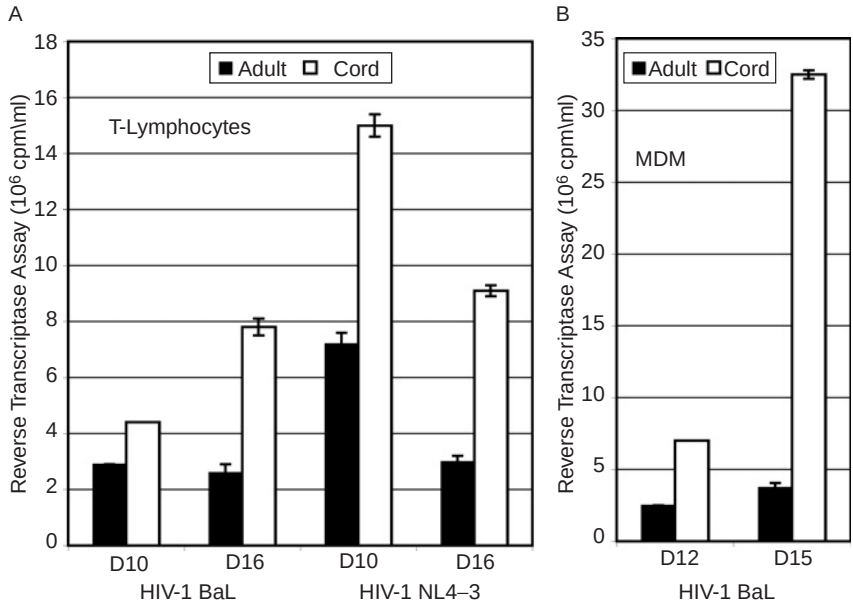


FIGURE 5 HIV-1 replication in T lymphocytes (A) and MDM (B) from seven different adult and neonatal (cord) blood donors. The results are expressed as RT assay (counts per million per milliliter ± SD) of triplicate experiments. HIV-1 replicates more efficiently (approximately threefold better) in cord T Lymphocytes and MDM (approximately ninefold better) compared with adult cells.

profound effect seen in MDM than T lymphocytes. To elucidate the mechanism of this differential HIV-1 replication in cord versus adult mononuclear cells, we determined the HIV-1 gene expression in cord and adult blood T lymphocytes and MDM by using a single-cycle replication competent pseudovirus, HIV-NL-Luc-E$^-$ (R^+/R^-) that measures transcriptional activity of HIV-1 LTR (Connor *et al*., 1995). Equal amounts of (1×10^5) RT counts of the NL-Luc-E$^-$ (R^+/R^-) viruses were used to infect T lymphocytes and MDM isolated from adult and cord blood, and HIV-1 gene expression was measured by luciferase activity (Connor *et al*., 1995). There was a threefold increase in luciferase activity in cord blood T lymphocytes (Fig. 6A) compared with adult T lymphocytes and a tenfold increase in cord blood MDM (Fig. 6B) compared with adult MDM (Sundaravaradan *et al*., 2006). The gene expression data here correlated with the data of HIV-1 replication kinetics (Sundaravaradan *et al*., 2006), suggesting that the increased gene expression of HIV-1 may contribute to an accelerated viral replication in cord blood target cells compared with adult cells. We observed donor-specific variability in the magnitude of HIV-1 gene expression in cord versus adult cells, with statistically significant higher levels of HIV-1 gene expression in cord versus adult cells. We conclude that the increased

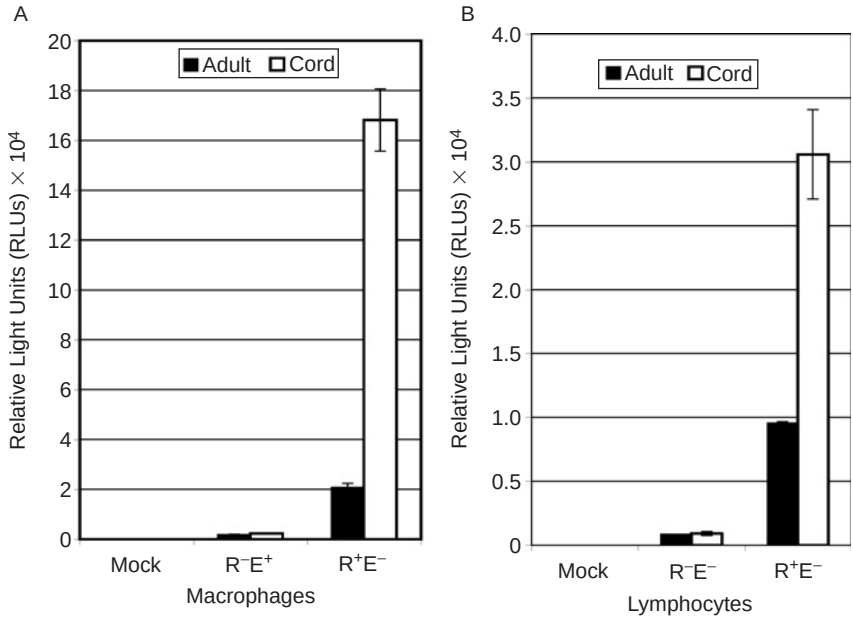


FIGURE 6 HIV-1 gene expression in T lymphocytes (A) and MDM (B) from seven different adult and cord blood donors. HIV-1 gene expression was measured as a function of luciferases expression driven by HIV-1 LTR in a single-cycle replication competent amphotropic envelope pseudovirus [HIV-NL-Luc-E$^-$ (R^+/R^-)] that measures transcriptional activity of HIV-1 LTR independent of the levels of HIV-1 receptor (CD4) and coreceptors (CCR5 or CXCR4) (Connor *et al*., 1995). The results are expressed as relative light units (RLUs) ± SD of triplicate experiments.

replication of HIV-1 seen in CBMCs is influenced at the level of gene expression, suggesting that HIV-1 LTR is being regulated differently in these cell types.

Although the neonatal immune system is not fully developed and is unable to contain the virus (Chakraborty, 2005; Rogers *et al*., 1987; Tiemessen and Kuhn, 2006), HIV-1 may interact differently with the immature immune cells and produce more HIV-1 than mature immune cells. It is likely that neonatal cells may express higher levels of transcriptional factors (Kedar *et al*., 1997), lower level of repressors, and/or differential levels of cellular factors than adult cells, which may be responsible for an increased HIV-1 gene expression in neonatal cells. The data presented in this chapter on increased HIV-1 infection in neonatal target cells may contribute to higher levels of viremia (Abrams *et al*., 1998; Henrard *et al*., 1995) and faster disease progression in infants compared with adults (MaWhinney *et al*., 1993; Tovo *et al*., 1992). These results provide new insights into the mechanisms of differential HIV-1 replication and disease progression in infants compared with adults.

XIV. The Future

With the use of ART during pregnancy, the risk of HIV-1 vertical transmission has been significantly reduced in developed countries. However, this treatment is not readily available in developing countries where most of the HIV-1 vertical transmission cases occur. One major concern is the long-term effect of ART on the development of uninfected children born to these treated infected mothers, which should be evaluated in several cohorts. Furthermore, better, effective, and new antivirals and a preventive vaccine should be developed to achieve a global prevention strategy of HIV-1 vertical transmission and infection. Several studies have shown a selective transmission of HIV-1 from mother to infant, including the biological properties of the transmitted viruses to be R5 utilizing CCR5 coreceptor. We have also characterized several molecular features of HIV-1 in several genes that may be associated with vertical transmission. In addition, we have shown that HIV-1 sequences from nontransmitting mothers are less heterogeneous than transmitting mothers. We have shown that HIV-1 replicates more efficiently in neonatal T lymphocytes and MDM compared with adult cells and this differential replication is significantly influenced at the level of HIV-1 gene expression. We should focus more on the understanding of the molecular mechanisms of HIV-1 vertical transmission and pathogenesis of HIV-1 infection in infants for developing new strategies for prevention and treatment of HIV-1 infection in infants.

Acknowledgments

I thank the National Institutes of Health (NIH/NIAID-AI40378, AI40378-06, NIH/FIC-TW01345) and Arizona Biomedical Research Commission (ADCRC-9601, 7002, 8001) for funding the grants and the University of Arizona for providing the facilities, environment, and support that allowed the accomplishment of the work described in this chapter. Dr. Colombe Chappey, Virologic, Inc., San Francisco, CA, and Nirav Merchant, University of Arizona Biotechnology Center, are acknowledged for assistance in nucleotide sequence analysis; Dr. Raymond C. Baker, Department of General Pediatrics, Children Hospital Medical Center, Cincinnati, OH, and Dr. Ziad M. Shehab, Department of Pediatrics, College of Medicine, University of Arizona, for providing HIV-1-infected mother–infant pairs' samples; AIDS Research and Reference Reagent Program for providing reagents, cell lines, and viral isolates and Dr. John J. Marchalonis, Chairman, Department of Microbiology & Immunology, University of Arizona, for support, encouragement, and fruitful discussions. Thanks to my past coworkers, Erik Matala, V. R. K. Yedavalli, Tobias Hahn, Mohammad Husain, Rajesh Ramakrishnan, Vasudha Sundaravaradan, Shailendra Saxena, Andrew Harodner, David Brooks, Kamlesh Patel, Scott Martin, Alison Holzer, and Tiffany Davis, and current coworkers, Roshni Mehta and Brian Wellensiek, for their contribution toward the ongoing research in the laboratory. My special thanks to Ms. Roshni Mehta for her assistance in the preparation of this manuscript.

References

Abrams, E. J., Weedon, J., Steketee, R. W., Lambert, G., Bamji, M., Brown, T., Kalish, M. L., Schoenbaum, E. E., Thomas, P. A., and Thea, D. M. (1998). Association of human immunodeficiency virus (HIV) load early in life with disease progression among HIV-infected infants. New York City Perinatal HIV Transmission Collaborative Study Group. *J. Infect. Dis.* **178**, 101–108.

Adjorlolo-Johnson, G., De Cock, K. M., Ekpini, E., Vetter, K. M., Sibailly, T., Brattegaard, K., Yavo, D., Doorly, R., Whitaker, J. P., Kestens, L., Ou, C.-Y., George, J. R., *et al.* (1994). Prospective comparison of mother-to-child transmission of HIV-1 and HIV-2 in Abidjan, Ivory Coast. *JAMA* **272**, 462–466.

Ahmad, N. (1996). Maternal-fetal transmission of human immunodeficiency virus. *J. Biomed. Sci.* **3**, 238–250.

Ahmad, N. (2000). Molecular mechanisms of human immunodeficiency virus type 1 mother-infant transmission. *Adv. Pharmacol.* **49**, 387–416.

Ahmad, N. (2005). The vertical transmission of human immunodeficiency virus type 1: Molecular and biological properties of the virus. *Crit. Rev. Clin. Lab. Sci.* **42**, 1–34.

Ahmad, N., and Venkatesan, S. (1988). Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science* **241**, 1481–1485.

Ahmad, N., Maitra, R. K., and Venkatesan, S. (1989). Rev-induced modulation of Nef protein underlies temporal regulation of human immunodeficiency virus replication. *Proc. Natl. Acad. Sci. USA* **86**, 6111–6115.

Ahmad, N., Baroudy, B. M., Baker, R. C., and Chappey, C. (1995). Genetic analysis of human immunodeficiency virus type 1 envelope V3 region isolates from mothers and infants after perinatal transmission. *J. Virol.* **69**, 1001–1012.

Ahmad, N., Matala, E., Yedavalli, R. K. V., Hahn, T., and Husain, M. (2000). Chacterization of human immunodeficiency virus type-1 involved in maternal-fetal transmission. *In* "Advances in Animal Virology" (S. Jameel and L. Villarreal, Eds.), pp. 351–370. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi.

Akari, H., Arold, S., Fukumori, T., Okazaki, T., Strebel, K., and Adachi, A. (2000). Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. *J. Virol.* **74**, 2907–2912.

Aldrovandi, G. M., Gao, L., Bristol, G., and Zack, J. A. (1998). Regions of human immunodeficiency virus type 1 nef required for function *in vivo*. *J. Virol.* **72**, 7032–7039.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996). CC CKR5: A RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.

Amadori, A., de Rossi, A., Giaquinto, C., Faulkner-Valle, G., Zacchello, F., and Chieco-Bianchi, L. (1988). *In-vitro* production of HIV-specific antibody in children at risk of AIDS. *Lancet* **1**, 852–854.

Amedee, A. M., Lacour, N., Gierman, J. L., Martin, L. N., Clements, J. E., Bohm, R., Jr., Harrison, R. M., and Murphey-Corb, M. (1995). Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. *J. Virol.* **69**, 7982–7990.

Auger, I., Thomas, P., De Gruttola, V., Morse, D., Moore, D., Williams, R., Truman, B., and Lawrence, C. E. (1988). Incubation periods for paediatric AIDS patients. *Nature* **336**, 575–577.

Balotta, C., Vigano, A., Riva, C., Colombo, M. C., Salvaggio, A., de Pasquale, M. P., Crupi, L., Papagno, L., Galli, M., Moroni, M., and Principi, N. (1996). HIV type 1 phenotype correlates with the stage of infection in vertically infected children. *AIDS Res. Hum. Retroviruses* **12**, 1247–1253.

Bamji, M., Thea, D. M., Weedon, J., Krasinski, K., Matheson, P. B., Thomas, P., Lambert, G., Abrams, E. J., Steketee, R., and Heagarty, M. (1996). Prospective study of human immunodeficiency virus 1-related disease among 512 infants born to infected women in New York City. The New York City Perinatal HIV Transmission Collaborative Study Group. *Pediatr. Infect. Dis. J.* **15**, 891–898.

Battiste, J. L., Mao, H., Rao, N. S., Tan, R., Muhandiram, D. R., Kay, L. E., Frankel, A. D., and Williamson, J. R. (1996). Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. *Science* **273**, 1547–1551.

Baur, A. S., Sass, G., Laffert, B., Willbold, D., Cheng-Mayer, C., and Peterlin, B. M. (1997). The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase. *Immunity* **6**, 283–291.

Bawdon, R. E., Gravell, M., Hamilton, R., Sever, J., Miller, R., and Gibbs, C. J. (1994). Studies on the placental transfer of cell-free human immunodeficiency virus and p24 antigen in an *ex vivo* human placental model. *J. Soc. Gynecol. Investig.* **1**, 45–48.

Becquart, P., Hocini, H., Levy, M., Sepou, A., Kazatchkine, M. D., and Belec, L. (2000). Secretory anti-human immunodeficiency virus (HIV) antibodies in colostrum and breast milk are not a major determinant of the protection of early postnatal transmission of HIV. *J. Infect. Dis.* **181**, 532–539.

Blanche, S., Rouzioux, C., Moscato, M. L., Veber, F., Mayaux, M. J., Jacomet, C., Tricoire, J., Deville, A., Vial, M., Firtion, G., *et al.* (1989). A prospective study of infants born to women seropositive for human immunodeficiency virus type 1. HIV Infection in Newborns French Collaborative Study Group. *N. Engl. J. Med.* **320**, 1643–1648.

Bobbitt, K. R., Addo, M. M., Altfeld, M., Filzen, T., Onafuwa, A. A., Walker, B. D., and Collins, K. L. (2003). Rev activity determines sensitivity of HIV-1-infected primary T cells to CTL killing. *Immunity* **18**, 289–299.

Bour, S., Schubert, U., and Strebel, K. (1995). The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: Implications for the mechanism of degradation. *J. Virol.* **69**, 1510–1520.

Bryson, Y. J., Luzuriaga, K., Sullivan, J. L., and Wara, D. W. (1992). Proposed definitions for in utero versus intrapartum transmission of HIV-1. *N. Engl. J. Med.* **327**, 1246–1247.

Bulterys, M., Chao, A., Dushimimana, A., Kageruka, M., Nawrocki, P., and Saah, A. J. (1992). HIV-exposed twins. *Lancet* **339**, 628.

Bunders, M., Thorne, C., and Newell, M. L. (2005). Maternal and infant factors and lymphocyte, CD4 and CD8 cell counts in uninfected children of HIV-1-infected mothers. *AIDS* **19**, 1071–1079.

Burns, D. N., FitzGerald, G., Semba, R., Hershow, R., Zorrilla, C., Pitt, J., Hammill, H., Cooper, E. R., Fowler, M. G., and Landesman, S. (1999). Vitamin A deficiency and other nutritional indices during pregnancy in human immunodeficiency virus infection: Prevalence, clinical correlates, and outcome. Women and Infants Transmission Study Group. *Clin. Infect. Dis.* **29**, 328–334.

Buseyne, F., Scott-Algara, D., Corre, B., Porrot, F., Monchatre, E., Bellal, N., Burgard, M., Rouzioux, C., Blanche, S., and Riviere, Y. (2006). Poor recognition of HIV-1 Nef protein by CD8 T cells from HIV-1-infected children: Impact of age. *Virology* **354**, 271–279.

Calvelli, T. A., and Rubinstein, A. (1990). Pediatric HIV infection: A review. *Immunodefic. Rev.* **2**, 83–127.

Cao, Y., Krogstad, P., Korber, B. T., Koup, R. A., Muldoon, M., Macken, C., Song, J. L., Jin, Z., Zhao, J. Q., Clapp, S., Chen, I. S., Ho, D. D., *et al.* (1997). Maternal HIV-1 viral load and vertical transmission of infection: The Ariel Project for the prevention of HIV transmission from mother to infant. *Nat. Med.* **3**, 549–552.

Casper, C., Naver, L., Clevestig, P., Belfrage, E., Leitner, T., Albert, J., Lindgren, S., Ottenblad, C., Bohlin, A. B., Fenyo, E. M., and Ehrnst, A. (2002). Coreceptor change appears after immune deficiency is established in children infected with different HIV-1 subtypes. *AIDS Res. Hum. Retroviruses* **18**, 343–352.

Centers for Disease Control and Prevention (1992). HIV/AIDS Surveillance Report. Centers for Disease Control and Prevention.

Chakraborty, R. (2005). HIV-1 infection in children: A clinical and immunologic overview. *Curr. HIV Res.* **3**, 31–41.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273.

Chandwani, S., Greco, M. A., Mittal, K., Antoine, C., Krasinski, K., and Borkowsky, W. (1991). Pathology and human immunodeficiency virus expression in placentas of seropositive women. *J. Infect. Dis.* **163**, 1134–1138.

Cheng-Mayer, C., Seto, D., Tateno, M., and Levy, J. A. (1988). Biologic features of HIV-1 that correlate with virulence in the host. *Science* **240**, 80–82.

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P. A., and Mayer, A. J. (1999). Selection for neutralization resistance of the simian/human immunodeficiency virus SHIVSF33A variant *in vivo* by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. *J. Virol.* **73**, 5294–5300.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., *et al.* (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135–1148.

Chouquet, C., Burgard, M., Richardson, S., Rouzioux, C., and Costagliola, D. (1997). Timing of mother-to-child HIV-1 transmission and diagnosis of infection based on polymerase chain reaction in the neonatal period by a non-parametric method. *AIDS* **11**, 1183–1184.

Chung, M. H., Kiarie, J. N., Richardson, B. A., Lehman, D. A., Overbaugh, J., and John-Stewart, G. C. (2005). Breast milk HIV-1 suppression and decreased transmission: A randomized trial comparing HIVNET 012 nevirapine versus short-course zidovudine. *AIDS* **19**, 1415–1422.

Cichutek, K., Merget, H., Norley, S., Linde, R., Kreuz, W., Gahr, M., and Kurth, R. (1992). Development of a quasispecies of human immunodeficiency virus type 1 *in vivo*. *Proc. Natl. Acad. Sci. USA* **89**, 7365–7369.

Clark, D. R., Ampel, N. M., Hallett, C. A., Yedavalli, V. R., Ahmad, N., and DeLuca, D. (1997). Peripheral blood from human immunodeficiency virus type 1-infected patients displays diminished T cell generation capacity. *J. Infect. Dis.* **176**, 649–654.

Cochrane, A. W., Perkins, A., and Rosen, C. A. (1990). Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: Relevance of nucleolar localization to function. *J. Virol.* **64**, 881–885.

Connor, R. I., and Ho, D. D. (1994). Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J. Virol.* **68**, 4400–4408.

Connor, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995). Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* **206**, 935–944.

Contag, C. H., Ehrnst, A., Duda, J., Bohlin, A. B., Lindgren, S., Learn, G. H., and Mullins, J. I. (1997). Mother-to-infant transmission of human immunodeficiency virus type 1 involving five envelope sequence subtypes. *J. Virol.* **71**, 1292–1300.

Cooper, E. R., Nugent, R. P., Diaz, C., Pitt, J., Hanson, C., Kalish, L. A., Mendez, H., Zorrilla, C., Hershow, R., Moye, J., Smeriglio, V., and Fowler, M. G. (1996). After AIDS clinical trial 076: The changing pattern of zidovudine use during pregnancy, and the subsequent reduction in the vertical transmission of human immunodeficiency virus in a cohort of infected women and their infants. Women and Infants Transmission Study Group. *J. Infect. Dis.* **174**, 1207–1211.

Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E., and Rusche, J. R. (1989). Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element *in vitro*. *Nature* **342**, 816–819.

Daly, T. J., Rennert, P., Lynch, P., Barry, J. K., Dundas, M., Rusche, J. R., Doten, R. C., Auer, M., and Farrington, G. K. (1993). Perturbation of the carboxy terminus of HIV-1 Rev affects multimerization on the Rev responsive element. *Biochemistry* **32**, 8945–8954.

Davison-Fairburn, B., Baskin, G., and Murphey-Corb, M. (1992). Maternal-fetal transmission of SIV in 2 species of monkeys. *In*: *Symposium Nonhuman Primate Models. AIDS* **10**, 29–35.

De Rossi, A., Ades, A. E., Mammano, F., Del Mistro, A., Amadori, A., Giaquinto, C., and Chieco-Bianchi, L. (1991). Antigen detection, virus culture, polymerase chain reaction, and *in vitro* antibody production in the diagnosis of vertically transmitted HIV-1 infection. *AIDS* **5**, 15–20.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., *et al.* (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**, 1856–1862.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W. A., Ratner, L., Kappes, J. C., Shaw, G. M., and Hunter, E. (2000). Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* **74**, 8358–8367.

Devash, Y., Calvelli, T. A., Wood, D. G., Reagan, K. J., and Rubinstein, A. (1990). Vertical transmission of human immunodeficiency virus is correlated with the absence of high-affinity/avidity maternal antibodies to the gp120 principal neutralizing domain. *Proc. Natl. Acad. Sci. USA* **87**, 3445–3449.

Di Marzio, P., Choe, S., Ebright, M., Knoblauch, R., and Landau, N. R. (1995). Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J. Virol.* **69**, 7909–7916.

di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (Marzo Veronese 1986). Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**, 1289–1291.

Dickover, R. E., Garratty, E. M., Herman, S. A., Sim, M. S., Plaeger, S., Boyer, P. J., Keller, M., Deveikis, A., Stiehm, E. R., and Bryson, Y. J. (1996). Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission. Effect of maternal zidovudine treatment on viral load. *JAMA* **275**, 599–605.

Dickover, R. E., Garratty, E. M., Plaeger, S., and Bryson, Y. J. (2001). Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants in utero and intrapartum. *J. Virol.* **75**, 2194–2203.

Ding, J., Jacobo-Molina, A., Tantillo, C., Lu, X., Nanni, R. G., and Arnold, E. (1994). Buried surface analysis of HIV-1 reverse transcriptase p66/p51 heterodimer and its interaction with dsDNA template/primer. *J. Mol. Recognit.* **7**, 157–161.

Ding, J., Hughes, S. H., and Arnold, E. (1997). Protein-nucleic acid interactions and DNA conformation in a complex of human immunodeficiency virus type 1 reverse transcriptase with a double-stranded DNA template-primer. *Biopolymers* **44**, 125–138.

Douglas, G. C., and King, B. F. (1992). Maternal-fetal transmission of human immunodeficiency virus: A review of possible routes and cellular mechanisms of infection. *Clin. Infect. Dis.* **15**, 678–691.

Dunn, D. T., Newell, M. L., Ades, A. E., and Peckham, C. S. (1992). Risk of human immunodeficiency virus type 1 transmission through breastfeeding. *Lancet* **340**, 585–588.

Ehrnst, A., Lindgren, S., Dictor, M., Johansson, B., Sonnerborg, A., Czajkowski, J., Sundin, G., and Bohlin, A. B. (1991). HIV in pregnant women and their offspring: Evidence for late transmission. *Lancet* **338**, 203–207.

Epstein, L. G., and Sharer, L. R. (1994). Neurological manifestations of perinatally acquired HIV-1 infection. *Semin. Pediatr. Neurol.* **1**, 50–57.

Fackler, O. T., d'Aloja, P., Baur, A. S., Federico, M., and Peterlin, B. M. (2001). Nef from human immunodeficiency virus type 1(F12) inhibits viral production and infectivity. *J. Virol.* **75**, 6601–6608.

Fang, G., Burger, H., Grimson, R., Tropper, P., Nachman, S., Mayers, D., Weislow, O., Moore, R., Reyelt, C., Hutcheon, N., Baker, D., and Weiser, B. (1995). Maternal plasma human immunodeficiency virus type 1 RNA level: A determinant and projected threshold for mother-to-child transmission. *Proc. Natl. Acad. Sci. USA* **92**, 12100–12104.

Fazely, F., Sharma, P. L., Fratazzi, C., Greene, M. F., Wyand, M. S., Memon, M. A., Penninck, D., and Ruprecht, R. M. (1993). Simian immunodeficiency virus infection via amniotic fluid: A model to study fetal immunopathogenesis and prophylaxis. *J. Acquir. Immune. Defic. Syndr.* **6**, 107–114.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.

Feng, Y. X., Campbell, S., Harvin, D., Ehresmann, B., Ehresmann, C., and Rein, A. (1999). The human immunodeficiency virus type 1 Gag polyprotein has nucleic acid chaperone activity: Possible role in dimerization of genomic RNA and placement of tRNA on the primer binding site. *J. Virol.* **73**, 4251–4256.

Freed, E. O. (1998). HIV-1 gag proteins: Diverse functions in the virus life cycle. *Virology* **251**, 1–15.

Frenkel, L. M., Wagner, L. E., 2nd, Demeter, L. M., Dewhurst, S., Coombs, R. W., Murante, B. L., and Reichman, R. C. (1995). Effects of zidovudine use during pregnancy on resistance and vertical transmission of human immunodeficiency virus type 1. *Clin. Infect. Dis.* **20**, 1321–1326.

Ganeshan, S., Dickover, R. E., Korber, B. T., Bryson, Y. J., and Wolinsky, S. M. (1997). Human immunodeficiency virus type 1 genetic evolution in children with different rates of development of disease. *J. Virol.* **71**, 663–677.

Garcia, P. M., Kalish, L. A., Pitt, J., Minkoff, H., Quinn, T. C., Burchett, S. K., Kornegay, J., Jackson, B., Moye, J., Hanson, C., Zorrilla, C., and Lew, J. F. (1999). Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Infants Transmission Study Group. *N. Engl. J. Med.* **341**, 394–402.

Geels, M. J., Cornelissen, M., Schuitemaker, H., Anderson, K., Kwa, D., Maas, J., Dekker, J. T., Baan, E., Zorgdrager, F., van den Burg, R., van Beelen, M., Lukashov, V. V., *et al.* (2003). Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual. *J. Virol.* **77**, 12430–12440.

Giaquinto, C., Rampon, O., and De Rossi, A. (2006). Antiretroviral therapy for prevention of mother-to-child HIV transmission: Focus on single-dose nevirapine. *Clin. Drug. Investig.* **26**, 611–627.

Global summary of AIDS epidemic (2006). UNAIDS/WHO AIDS epidemic update: December 2006. http://www.unaids.org/en/HIV_data/epi2006/

Goedert, J. J., Duliege, A. M., Amos, C. I., Felton, S., and Biggar, R. J. (1991). High risk of HIV-1 infection for first-born twins. The international registry of HIV-exposed twins. *Lancet* **338**, 1471–1475.

Goh, W. C., Rogel, M. E., Kinsey, C. M., Michael, S. F., Fultz, P. N., Nowak, M. A., Hahn, B. H., and Emerman, M. (1998). HIV-1 Vpr increases viral expression by manipulation of the cell cycle: A mechanism for selection of Vpr *in vivo*. *Nat. Med.* **4**, 65–71.

Gordon, M., De Oliveira, T., Bishop, K., Coovadia, H. M., Madurai, L., Engelbrecht, S., Janse van Rensburg, E., Mosam, A., Smith, A., and Cassol, S. (2003). Molecular characteristics of human immunodeficiency virus type 1 subtype C viruses from KwaZulu-Natal, South

Africa: Implications for vaccine and antiretroviral control strategies. *J. Virol.* **77**, 2587–2599.

Gotte, M., Li, X., and Wainberg, M. A. (1999). HIV-1 reverse transcription: A brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Arch. Biochem. Biophys.* **365**, 199–210.

Gotte, M., Maier, G., Gross, H. J., and Heumann, H. (1998). Localization of the active site of HIV-1 reverse transcriptase-associated RNase H domain on a DNA template using site-specific generated hydroxyl radicals. *J. Biol. Chem.* **273**, 10139–10146.

Goulder, P. J., Brander, C., Tang, Y., Tremblay, C., Colbert, R. A., Addo, M. M., Rosenberg, E. S., Nguyen, T., Allen, R., Trocha, A., Altfeld, M., He, S., *et al.* (2001). Evolution and transmission of stable CTL escape mutations in HIV infection*Nature* **412**, 334–338.

Grody, W. W., Fligiel, S., and Naeim, F. (1985). Thymus involution in the acquired immunodeficiency syndrome. *Am. J. Clin. Pathol.* **84**, 85–95.

Grzesiek, S., Stahl, S. J., Wingfield, P. T., and Bax, A. (1996). The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry* **35**, 10256–10261.

Guay, L. A., Musoke, P., Fleming, T., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Ducar, C., Deseyve, M., Emel, L., Mirochnick, M., *et al.* (1999). Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *Lancet* **354**, 795–802.

Hahn, T., and Ahmad, N. (2001). Genetic characterization of HIV type 1 gag p17 matrix genes in isolates from infected mothers lacking perinatal transmission. *AIDS Res. Hum. Retroviruses* **17**, 1673–1680.

Hahn, T., Matala, E., Chappey, C., and Ahmad, N. (1999). Characterization of mother-infant HIV type 1 gag p17 sequences associated with perinatal transmission. *AIDS Res. Hum. Retroviruses* **15**, 875–888.

Hahn, T., Ramakrishnan, R., and Ahmad, N. (2003). Evaluation of genetic diversity of human immunodeficiency virus type 1 NEF gene associated with vertical transmission. *J. Biomed. Sci.* **10**, 436–450.

Halsey, N. A., Markham, R., Wahren, B., Boulos, R., Rossi, P., and Wigzell, H. (1992). Lack of association between maternal antibodies to V3 loop peptides and maternal-infant HIV-1 transmission. *J. Acquir. Immune Defic. Syndr.* **5**, 153–157.

Hammerschmid, M., Palmeri, D., Ruhl, M., Jaksche, H., Weichselbraun, I., Bohnlein, E., Malim, M. H., and Hauber, J. (1994). Scanning mutagenesis of the arginine-rich region of the human immunodeficiency virus type 1 Rev trans activator. *J. Virol.* **68**, 7329–7335.

Harris, D., Kaushik, N., Pandey, P. K., Yadav, P. N., and Pandey, V. N. (1998). Functional analysis of amino acid residues constituting the dNTP binding pocket of HIV-1 reverse transcriptase. *J. Biol. Chem.* **273**, 33624–33634.

Heath, M. J., Derebail, S. S., Gorelick, R. J., and DeStefano, J. J. (2003). Differing roles of the N- and C-terminal zinc fingers in human immunodeficiency virus nucleocapsid protein-enhanced nucleic acid annealing. *J. Biol. Chem.* **278**, 30755–30763.

Henin, Y., Mandelbrot, L., Henrion, R., Pradinaud, R., Coulaud, J. P., and Montagnier, L. (1993). Virus excretion in the cervicovaginal secretions of pregnant and nonpregnant HIV-infected women. *J. Acquir. Immune Defic. Syndr.* **6**, 72–75.

Henrard, D. R., Phillips, J. F., Muenz, L. R., Blattner, W. A., Wiesner, D., Eyster, M. E., and Goedert, J. J. (1995). Natural history of HIV-1 cell-free viremia. *JAMA* **274**, 554–558.

Hira, S. K., Kamanga, J., Bhat, G. J., Mwale, C., Tembo, G., Luo, N., and Perine, P. L. (1989). Perinatal transmission of HIV-I in Zambia. *Br. Med. J.* **299**, 1250–1252.

Hoff, R., Berardi, V. P., Weiblen, B. J., Mahoney-Trout, L., Mitchell, M. L., and Grady, G. F. (1988). Seroprevalence of human immunodeficiency virus among childbearing women. Estimation by testing samples of blood from newborns. *N. Engl. J. Med.* **318**, 525–530.

Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug resistance. *Science* **282**, 1669–1675.

Husain, M., Hahn, T., Yedavalli, V. R., and Ahmad, N. (2001). Characterization of HIV type 1 tat sequences associated with perinatal transmission. *AIDS Res. Hum. Retroviruses* **17**, 765–773.

Hutto, C., Zhou, Y., He, J., Geffin, R., Hill, M., Scott, W., and Wood, C. (1996). Longitudinal studies of viral sequence, viral phenotype, and immunologic parameters of human immunodeficiency virus type 1 infection in perinatally infected twins with discordant disease courses. *J. Virol.* **70**, 3589–3598.

Hwang, S. S., Boyle, T. J., Lyerly, H. K., and Cullen, B. R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71–74.

Iafrate, A. J., Bronson, S., and Skowronski, J. (1997). Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling. *EMBO J.* **16**, 673–684.

Ibe, S., Hotta, N., Takeo, U., Tawada, Y., Mamiya, N., Yamanaka, K., Utsumi, M., and Kaneda, T. (2003a). Prevalence of drug-resistant human immunodeficiency virus type 1 in therapy-naive patients and usefulness of genotype testing. *Microbiol. Immunol.* **47**, 499–505.

Ibe, S., Shibata, N., Utsumi, M., and Kaneda, T. (2003b). Selection of human immunodeficiency virus type 1 variants with an insertion mutation in the p6(gag) and p6(pol) genes under highly active antiretroviral therapy. *Microbiol. Immunol.* **47**, 71–79.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S., *et al.* (1993). Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**, 6320–6324.

Jain, C., and Belasco, J. G. (2001). Structural model for the cooperative assembly of HIV-1 Rev multimers on the RRE as deduced from analysis of assembly-defective mutants. *Mol. Cell.* **7**, 603–614.

Jameel, S., Zafrullah, M., Ahmad, M., Kapoor, G. S., and Sehgal, S. (1995). A genetic analysis of HIV-1 from Punjab, India reveals the presence of multiple variants. *AIDS* **9**, 685–690.

Jeang, K. T., Xiao, H., and Rich, E. A. (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J. Biol. Chem.* **274**, 28837–28840.

John, G. C., Rousseau, C., Dong, T., Rowland-Jones, S., Nduati, R., Mbori-Ngacha, D., Rostron, T., Kreiss, J. K., Richardson, B. A., and Overbaugh, J. (2000). Maternal SDF1 3′A polymorphism is associated with increased perinatal human immunodeficiency virus type 1 transmission. *J. Virol.* **74**, 5736–5739.

Joshi, V. V., and Oleske, J. M. (1985). Pathologic appraisal of the thymus gland in acquired immunodeficiency syndrome in children. A study of four cases and a review of the literature. *Arch. Pathol. Lab. Med.* **109**, 142–146.

Julias, J. G., McWilliams, M. J., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2002). Mutations in the RNase H domain of HIV-1 reverse transcriptase affect the initiation of DNA synthesis and the specificity of RNase H cleavage in vivo. *Proc. Natl. Acad. Sci. USA* **99**, 9515–9520.

Julias, J. G., McWilliams, M. J., Sarafianos, S. G., Alvord, W. G., Arnold, E., and Hughes, S. H. (2003). Mutation of amino acids in the connection domain of human immunodeficiency virus type 1 reverse transcriptase that contact the template-primer affects RNase H activity. *J. Virol.* **77**, 8548–8554.

Kao, S. M., Miller, E. D., and Su, L. (2001). A leucine zipper motif in the cytoplasmic domain of gp41 is required for HIV-1 replication and pathogenesis *in vivo*. *Virology* **289**, 208–217.

Kedar, P. S., Arden, K., Foyle, M., Pope, J. H., and Zeichner, S. L. (1997). Umbilical cord blood mononuclear cell HIV-1 LTR Binding Activities. *J. Biomed. Sci.* **4**, 217–228.

Kimura, T., Nishikawa, M., and Ohyama, A. (1994). Intracellular membrane traffic of human immunodeficiency virus type 1 envelope glycoproteins: Vpu liberates Golgi-targeted gp160 from CD4-dependent retention in the endoplasmic reticulum. *J Biochem (Tokyo)* **115**, 1010–1020.

Kitchen, S. G., and Zack, J. A. (1997). CXCR4 expression during lymphopoiesis: Implications for human immunodeficiency virus type 1 infection of the thymus. *J. Virol.* **71**, 6928–6934.

Kliks, S. C., Wara, D. W., Landers, D. V., and Levy, J. A. (1994). Features of HIV-1 that could influence maternal-child transmission. *JAMA* **272**, 467–474.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.

Koito, A., Harrowe, G., Levy, J. A., and Cheng-Mayer, C. (1994). Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and soluble CD4 neutralization. *J. Virol.* **68**, 2253–2259.

Korber, B., Brander, C., Haynes, B. F., Koup, R. A., Moore, J., Walker, B. D., and Watkins, D. I. (2003). HIV Immunology and HIV/SIV Vaccine Databases.

Koup, R. A., and Wilson, C. B. (1993). Clinical immunology of HIV-infected children. *In* "Pediatric AIDS: The Challenge of HIV Infection in Infants, Children, and Adolescents" (P. A. Pizzo and C. M. Wilfert, Eds.), pp. 158–165. Williams and Wilkins, Baltimore, Maryland.

Kourtis, A. P., Lee, F. K., Abrams, E. J., Jamieson, D. J., and Bulterys, M. (2006). Mother-to-child transmission of HIV-1: Timing and implications for prevention. *Lancet. Infect. Dis.* **6**, 726–732.

Koyanagi, Y., Miles, S., Mitsuyasu, R. T., Merrill, J. E., Vinters, H. V., and Chen, I. S. (1987). Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science* **236**, 819–822.

Krishnamoorthy, G., Roques, B., Darlix, J. L., and Mely, Y. (2003). DNA condensation by the nucleocapsid protein of HIV-1: A mechanism ensuring DNA protection. *Nucleic Acids Res.* **31**, 5425–5432.

Krivine, A., Firtion, G., Cao, L., Francoual, C., Henrion, R., and Lebon, P. (1992). HIV replication during the first weeks of life. *Lancet* **339**, 1187–1189.

Kuiken, C., Foley, B., Guzman, E., Korber, B., and Crandall, K. A. (1999). *The Evolution of HIV.* The John Hopinks University Press, Baltimore.

Labrosse, B., Labernardiere, J. L., Dam, E., Trouplin, V., Skrabal, K., Clavel, F., and Mammano, F. (2003). Baseline susceptibility of primary human immunodeficiency virus type 1 to entry inhibitors. *J. Virol.* **77**, 1610–1613.

Lairmore, M. D., Cuthbert, P. S., Utley, L. L., Morgan, C. J., Dezzutti, C. S., Anderson, C. L., and Sedmak, D. D. (1993). Cellular localization of CD4 in the human placenta. Implications for maternal-to-fetal transmission of HIV. *J. Immunol.* **151**, 1673–1681.

Lamers, S. L., Sleasman, J. W., She, J. X., Barrie, K. A., Pomeroy, S. M., Barrett, D. J., and Goodenow, M. M. (1994). Persistence of multiple maternal genotypes of human immunodeficiency virus type I in infants infected by vertical transmission. *J. Clin. Invest.* **93**, 380–390.

Laure, F., Courgnaud, V., Rouzioux, C., Blanche, S., Veber, F., Burgard, M., Jacomet, C., Griscelli, C., and Brechot, C. (1988). Detection of HIV1 DNA in infants and children by means of the polymerase chain reaction. *Lancet* **2**, 538–541.

Lepage, P., Van de Perre, P., Carael, M., Nsengumuremyi, F., Nkurunziza, J., Butzler, J. P., and Sprecher, S. (1987). Postnatal transmission of HIV from mother to child. *Lancet* **2**, 400.

Lohman, B. L., Slyker, J. A., Richardson, B. A., Farquhar, C., Mabuka, J. M., Crudder, C., Dong, T., Obimbo, E., Mbori-Ngacha, D., Overbaugh, J., Rowland-Jones, S., and John-Stewart, G. (2005). Longitudinal assessment of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon responses during the first year of life in HIV-1-infected infants. *J. Virol.* **79**, 8121–8130.

Lu, M., Stoller, M. O., Wang, S., Liu, J., Fagan, M. B., and Nunberg, J. H. (2001). Structural and functional analysis of interhelical interactions in the human immunodeficiency virus type 1 gp41 envelope glycoprotein by alanine-scanning mutagenesis. *J. Virol.* **75**, 11146–11156.

Lyman, W. D., Kress, Y., Rashbaum, W. K., Calvelli, T. A., Steinhauer, E., Kashkin, J. M., Henderson, C. E., and Rubinstein, A. (1988). An AIDS virus-associated antigen localized in human fetal brain. *Ann. NY. Acad. Sci.* **540**, 628–629.

Macreadie, I. G., Castelli, L. A., Hewish, D. R., Kirkpatrick, A., Ward, A. C., and Azad, A. A. (1995). A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. *Proc. Natl. Acad. Sci. USA* **92**, 2770–2774.

Mahalingam, S., Khan, S. A., Jabbar, M. A., Monken, C. E., Collman, R. G., and Srinivasan, A. (1995). Identification of residues in the N-terminal acidic domain of HIV-1 Vpr essential for virion incorporation. *Virology* **207**, 297–302.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., and Weiner, D. B. (1997). Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. *J. Virol.* **71**, 6339–6347.

Malim, M. H., Bohnlein, S., Hauber, J., and Cullen, B. R. (1989). Functional dissection of the HIV-1 Rev trans-activator—derivation of a trans-dominant repressor of Rev function. *Cell* **58**, 205–214.

Malim, M. H., McCarn, D. F., Tiley, L. S., and Cullen, B. R. (1991). Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. *J. Virol.* **65**, 4248–4254.

Mandal, D., Jana, S., Bhattacharya, S. K., and Chakrabarti, S. (2002). HIV type 1 subtypes circulating in eastern and northeastern regions of India. *AIDS Res. Hum. Retroviruses* **18**, 1219–1227.

Mangasarian, A., Piguet, V., Wang, J. K., Chen, Y. L., and Trono, D. (1999). Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. *J. Virol.* **73**, 1964–1973.

Matala, E., Crandall, K. A., Baker, R. C., and Ahmad, N. (2000). Limited heterogeneity of HIV type 1 in infected mothers correlates with lack of vertical transmission. *AIDS Res. Hum. Retroviruses* **16**, 1481–1489.

Matala, E., Hahn, T., Yedavalli, V. R., and Ahmad, N. (2001). Biological characterization of HIV type 1 envelope V3 regions from mothers and infants associated with perinatal transmission. *AIDS Res. Hum. Retroviruses* **17**, 1725–1735.

Matthews, T. J., Langlois, A. J., Robey, W. G., Chang, N. T., Gallo, R. C., Fischinger, P. J., and Bolognesi, D. P. (1986). Restricted neutralization of divergent human T-lymphotropic virus type III isolates by antibodies to the major envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **83**, 9709–9713.

Maury, W., Potts, B. J., and Rabson, A. B. (1989). HIV-1 infection of first-trimester and term human placental tissue: A possible mode of maternal-fetal transmission. *J. Infect. Dis.* **160**, 583–588.

MaWhinney, S., Pagano, M., and Thomas, P. (1993). Age at AIDS diagnosis for children with perinatally acquired HIV. *J. Acquir. Immune Defic. Syndr.* **6**, 1139–1144.

Mayaux, M. J., Blanche, S., Rouzioux, C., Le Chenadec, J., Chambrin, V., Firtion, G., Allemon, M. C., Vilmer, E., Vigneron, N. C., Tricoire, J., Guillot, F., Courpotin, C., and French Pediatric HIV Infection Study Group (1995). Maternal factors associated with perinatal HIV-1 transmission: The French Cohort Study: 7 years of follow-up observation. The French Pediatric HIV Infection Study Group. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **8**, 188–194.

McClure, H. M., Anderson, D. C., Fultz, P. N., Ansari, A. A., Jehuda-Cohen, T., Villinger, F., Klumpp, S. A., Switzer, W., Lockwood, E., Brodie, A., *et al.* (1991). Maternal transmission of SIVsmm in rhesus macaques. *J. Med. Primatol.* **20**, 182–187.

McCormick-Davis, C., Dalton, S. B., Singh, D. K., and Stephens, E. B. (2000). Comparison of Vpu sequences from diverse geographical isolates of HIV type 1 identifies the presence of highly variable domains, additional invariant amino acids, and a signature sequence motif common to subtype C isolates. *AIDS Res. Hum. Retroviruses* **16**, 1089–1095.

McGowan, J. P., Crane, M., Wiznia, A. A., and Blum, S. (1999). Combination antiretroviral therapy in human immunodeficiency virus-infected pregnant women. *Obstet. Gynecol.* **94**, 641–646.

McNearney, T., Westervelt, P., Thielan, B. J., Trowbridge, D. B., Garcia, J., Whittier, R., and Ratner, L. (1990). Limited sequence heterogeneity among biologically distinct human immunodeficiency virus type 1 isolates from individuals involved in a clustered infectious outbreak. *Proc. Natl. Acad. Sci. USA* **87**, 1917–1921.

Menendez-Arias, L., Mas, A., and Domingo, E. (1998). Cytotoxic T-lymphocyte responses to HIV-1 reverse transcriptase (review). *Viral Immunol.* **11**, 167–181.

Minkoff, H., and Augenbraun, M. (1997). Antiretroviral therapy for pregnant women. *Am. J. Obstet. Gynecol.* **176**, 478–489.

Miyauchi, K., Komano, J., Yokomaku, Y., Sugiura, W., Yamamoto, N., and Matsuda, Z. (2005). Role of the specific amino acid sequence of the membrane-spanning domain of human immunodeficiency virus type 1 in membrane fusion. *J. Virol.* **79**, 4720–4729.

Mo, H., Monard, S., Pollack, H., Ip, J., Rochford, G., Wu, L., Hoxie, J., Borkowsky, W., Ho, D. D., and Moore, J. P. (1998). Expression patterns of the HIV type 1 coreceptors CCR5 and CXCR4 on CD4+ T cells and monocytes from cord and adult blood. *AIDS Res. Hum. Retroviruses* **14**, 607–617.

Mok, J. Q., Giaquinto, C., De Rossi, A., Grosch-Worner, I., Ades, A. E., and Peckham, C. S. (1987). Infants born to mothers seropositive for human immunodeficiency virus. Preliminary findings from a multicentre European study. *Lancet* **1**, 1164–1168.

Moodley, J., and Wennberg, J. L. (2005). HIV in pregnancy. *Curr. Opin. Obstet. Gynecol.* **17**, 117–121.

Mulder-Kampinga, G. A., Kuiken, C., Dekker, J., Scherpbier, H. J., Boer, K., and Goudsmit, J. (1993). Genomic human immunodeficiency virus type 1 RNA variation in mother and child following intra-uterine virus transmission. *J. Gen. Virol.* **74**(Pt 9), 1747–1756.

Mulder-Kampinga, G. A., Simonon, A., Kuiken, C. L., Dekker, J., Scherpbier, H. J., van de Perre, P., Boer, K., and Goudsmit, J. (1995). Similarity in env and gag genes between genomic RNAs of human immunodeficiency virus type 1 (HIV-1) from mother and infant is unrelated to time of HIV-1 RNA positivity in the child. *J. Virol.* **69**, 2285–2296.

Mulky, A., Sarafianos, S. G., Arnold, E., Wu, X., and Kappes, J. C. (2004). Subunit-specific analysis of the human immunodeficiency virus type 1 reverse transcriptase *in vivo*. *J. Virol.* **78**, 7089–7096.

Myers, G., Korber, B., and Hahn, B. H. (1995). Human retroviruses and AIDS database. Theoretical Biology, Los Alamos National Laboratory: Los Alamos.

Nair, P., Alger, L., Hines, S., Seiden, S., Hebel, R., and Johnson, J. P. (1993). Maternal and neonatal characteristics associated with HIV infection in infants of seropositive women. *J. Acquir. Immune Defic. Syndr.* **6**, 298–302.

Narwa, R., Roques, P., Courpotin, C., Parnet-Mathieu, F., Boussin, F., Roane, A., Marce, D., Lasfargues, G., and Dormont, D. (1996). Characterization of human immunodeficiency virus type 1 p17 matrix protein motifs associated with mother-to-child transmission. *J. Virol.* **70**, 4474–4483.

Nduati, R., John, G., Mbori-Ngacha, D., Richardson, B., Overbaugh, J., Mwatha, A., Ndinya-Achola, J., Bwayo, J., Onyango, F. E., Hughes, J., and Kreiss, J. (2000). Effect of

breastfeeding and formula feeding on transmission of HIV-1: A randomized clinical trial. *JAMA* **283**, 1167–1174.

Nduati, R. W., John, G. C., Richardson, B. A., Overbaugh, J., Welch, M., Ndinya-Achola, J., Moses, S., Holmes, K., Onyango, F., and Kreiss, J. K. (1995). Human immunodeficiency virus type 1-infected cells in breast milk: Association with immunosuppression and vitamin A deficiency. *J. Infect. Dis.* **172**, 1461–1468.

Newell, M. L., and Peckham, C. (1993). Risk factors for vertical transmission of HIV-1 and early markers of HIV-1 infection in children. *AIDS* **7**(Suppl. 1), S91–S97.

Nicholas, S. W., Sondheimer, D. L., Willoughby, A. D., Yaffe, S. J., and Katz, S. L. (1989). Human immunodeficiency virus infection in childhood, adolescence, and pregnancy: A status report and national research agenda. *Pediatrics* **83**, 293–308.

Oberste, M. S., and Gonda, M. A. (1992). Conservation of amino-acid sequence motifs in lentivirus Vif proteins. *Virus Genes* **6**, 95–102.

Oelrichs, R. B., Shrestha, I. L., Anderson, D. A., and Deacon, N. J. (2000). The explosive human immunodeficiency virus type 1 epidemic among injecting drug users of Kathmandu, Nepal, is caused by a subtype C virus of restricted genetic diversity. *J. Virol.* **74**, 1149–1157.

Ohagen, A., Devitt, A., Kunstman, K. J., Gorry, P. R., Rose, P. P., Korber, B., Taylor, J., Levy, R., Murphy, R. L., Wolinsky, S. M., and Gabuzda, D. (2003). Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. *J. Virol.* **77**, 12336–12345.

Oleske, J., Minnefor, A., Cooper, R., Jr., Thomas, K., dela Cruz, A., Ahdieh, H., Guerrero, I., Joshi, V. V., and Desposito, F. (1983). Immune deficiency syndrome in children. *JAMA* **249**, 2345–2349.

Olsen, H. S., Beidas, S., Dillon, P., Rosen, C. A., and Cochrane, A. W. (1991). Mutational analysis of the HIV-1 Rev protein and its target sequence, the Rev responsive element. *J. Acquir. Immune Defic. Syndr.* **4**, 558–567.

Parekh, B. S., Shaffer, N., Pau, C. P., Abrams, E., Thomas, P., Pollack, H., Bamji, M., Kaul, A., Schochetman, G., Rogers, M., George, J. R., and NYC, Collaborative Group (1991).Lack of correlation between maternal antibodies to V3 loop peptides of gp120 and perinatal HIV-1 transmission. The NYC Perinatal HIV Transmission Collaborative Study. *AIDS* **5**, 1179–1184.

Pasquier, C., Cayrou, C., Blancher, A., Tourne-Petheil, C., Berrebi, A., Tricoire, J., Puel, J., and Izopet, J. (1998). Molecular evidence for mother-to-child transmission of multiple variants by analysis of RNA and DNA sequences of human immunodeficiency virus type 1. *J. Virol.* **72**, 8493–8501.

Peters, S., Munoz, M., Yerly, S., Sanchez-Merino, V., Lopez-Galindez, C., Perrin, L., Larder, B., Cmarko, D., Fakan, S., Meylan, P., and Telenti, A. (2001). Resistance to nucleoside analog reverse transcriptase inhibitors mediated by human immunodeficiency virus type 1 p6 protein. *J. Virol.* **75**, 9644–9653.

Petropoulou, H., Stratigos, A. J., and Katsambas, A. D. (2006). Human immunodeficiency virus infection and pregnancy. *Clin. Dermatol.* **24**, 536–542.

Philpott, S., Burger, H., Charbonneau, T., Grimson, R., Vermund, S. H., Visosky, A., Nachman, S., Kovacs, A., Tropper, P., Frey, H., and Weiser, B. (1999). CCR5 genotype and resistance to vertical transmission of HIV-1. *J. Acquir. Immune Defic. Syndr.* **21**, 189–193.

Poon, D. T., Wu, J., and Aldovini, A. (1996). Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity. *J. Virol.* **70**, 6607–6616.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R., and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. USA* **94**, 1890–1895.

Putney, S. D., Matthews, T. J., Robey, W. G., Lynn, D. L., Robert-Guroff, M., Mueller, W. T., Langlois, A. J., Ghrayeb, J., Petteway, S. R., Jr., Weinhold, K. J., Fischinger, P. J., WongStaal, F., *et al.* (1986). HTLV-III/LAV-neutralizing antibodies to an E. coli-produced fragment of the virus envelope. *Science* **234**, 1392–1395.

Ramakrishnan, R., Hussain, M., Holzer, A., Mehta, R., Sundaravaradan, V., and Ahmad, N. (2005). Evaluations of HIV type 1 rev gene diversity and functional domains following perinatal transmission. *AIDS Res. Hum. Retroviruses* **21**, 1035–1045.

Ramakrishnan, R., Mehta, R., Sundaravaradan, V., Davis, T., and Ahmad, N. (2006). Characterization of HIV-1 envelope gp41 genetic diversity and functional domains following perinatal transmission. *Retrovirology* **3**, 42.

Rana, T. M., and Jeang, K. T. (1999). Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA. *Arch. Biochem. Biophys.* **365**, 175–185.

Reeves, J. D., Gallo, S. A., Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E., and Doms, R. W. (2002). Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc. Natl. Acad. Sci. USA* **99**, 16249–16254.

Reitter, J. N., Means, R. E., and Desrosiers, R. C. (1998). A role for carbohydrates in immune evasion in AIDS. *Nat. Med.* **4**, 679–684.

Report of a Consensus Workshop (1992). Report of a Consensus Workshop, Siena, Italy, January 17–18, 1992. Maternal factors involved in mother-to-child transmission of HIV-1. *J. Acquir. Immune Defic. Syndr.* **5**, 1019–1029.

Resino, S., Gurbindo, D., Cano, J. M., Sanchez-Ramon, S., and Muoz-Fernandez, M. A. (2000). Predictive markers of clinical outcome in vertically HIV-1-infected infants. A prospective longitudinal study. *Pediatr. Res.* **47**, 509–515.

Richman, D. D., and Bozzette, S. A. (1994). The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. *J. Infect. Dis.* **169**, 968–974.

Rogers, M. F., Ou, C. Y., Rayfield, M., Thomas, P. A., Schoenbaum, E. E., Abrams, E., Krasinski, K., Selwyn, P. A., Moore, J., Kaul, A., *et al.* (1989). Use of the polymerase chain reaction for early detection of the proviral sequences of human immunodeficiency virus in infants born to seropositive mothers. New York City Collaborative Study of Maternal HIV Transmission and Montefiore Medical Center HIV Perinatal Transmission Study Group. *N. Engl. J. Med.* **320**, 1649–1654.

Rogers, M. F., Thomas, P. A., Starcher, E. T., Noa, M. C., Bush, T. J., and Jaffe, H. W. (1987). Acquired immunodeficiency syndrome in children: Report of the Centers for Disease Control National Surveillance, 1982 to 1985. *Pediatrics* **79**, 1008–1014.

Rosenzweig, M., Clark, D. P., and Gaulton, G. N. (1993). Selective thymocyte depletion in neonatal HIV-1 thymic infection. *AIDS* **7**, 1601–1605.

Rossi, P., Moschese, V., Broliden, P. A., Fundaro, C., Quinti, I., Plebani, A., Giaquinto, C., Tovo, P. A., Ljunggren, K., Rosen, J., Wigzell, H., Jondal, M., *et al.* (1989). Presence of maternal antibodies to human immunodeficiency virus 1 envelope glycoprotein gp120 epitopes correlates with the uninfected status of children born to seropositive mothers. *Proc. Natl. Acad. Sci. USA* **86**, 8055–8058.

Rousseau, C. M., Nduati, R. W., Richardson, B. A., John-Stewart, G. C., Mbori-Ngacha, D. A., Kreiss, J. K., and Overbaugh, J. (2004). Association of levels of HIV-1-infected breast milk cells and risk of mother-to-child transmission. *J. Infect. Dis.* **190**, 1880–1888.

Rouzioux, C., Costagliola, D., Burgard, M., Blanche, S., Mayaux, M. J., Griscelli, C., and Valleron, A. J. (1993). Timing of mother-to-child HIV-1 transmission depends on maternal status. The HIV Infection in Newborns French Collaborative Study Group. *AIDS* **7**(Suppl. 2), S49–S52.

Rowland-Jones, S. L., Phillips, R. E., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R., *et al.* (1992). Human

immunodeficiency virus variants that escape cytotoxic T-cell recognition. *AIDS Res. Hum. Retroviruses* **8**, 1353–1354.

Rubinstein, A., Sicklick, M., Gupta, A., Bernstein, L., Klein, N., Rubinstein, E., Spigland, I., Fruchter, L., Litman, N., Lee, H., and Hollander, M. (1983). Acquired immunodeficiency with reversed T4/T8 ratios in infants born to promiscuous and drug-addicted mothers. *JAMA* **249**, 2350–2356.

Rusche, J. R., Javaherian, K., McDanal, C., Petro, J., Lynn, D. L., Grimaila, R., Langlois, A., Gallo, R. C., Arthur, L. O., Fischinger, P. J., Bolognesi, D. P., Putney, S. D., *et al.* (1988). Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. *Proc. Natl. Acad. Sci. USA* **85**, 3198–3202.

Ryder, R. W., Nsa, W., Hassig, S. E., Behets, F., Rayfield, M., Ekungola, B., Nelson, A. M., Mulenda, U., Francis, H., Mwandagalirwa, K., Davachi, F., Rogers, M., *et al.* (1989). Perinatal transmission of the human immunodeficiency virus type 1 to infants of seropositive women in Zaire. *N. Engl. J. Med.* **320**, 1637–1642.

Saksela, K., Cheng, G., and Baltimore, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J.* **14**, 484–491.

Sato, H., Shiino, T., Kodaka, N., Taniguchi, K., Tomita, Y., Kato, K., Miyakuni, T., and Takebe, Y. (1999). Evolution and biological characterization of human immunodeficiency virus type 1 subtype E gp120 V3 sequences following horizontal and vertical virus transmission in a single family. *J. Virol.* **73**, 3551–3559.

Scarlatti, G., Albert, J., Rossi, P., Hodara, V., Biraghi, P., Muggiasca, L., and Fenyo, E. M. (1993a). Mother-to-child transmission of human immunodeficiency virus type 1: Correlation with neutralizing antibodies against primary isolates. *J. Infect. Dis.* **168**, 207–210.

Scarlatti, G., Leitner, T., Halapi, E., Wahlberg, J., Marchisio, P., Clerici-Schoeller, M. A., Wigzell, H., Fenyo, E. M., Albert, J., Uhlen, M., and Rossi, P. (1993b).Comparison of variable region 3 sequences of human immunodeficiency virus type 1 from infected children with the RNA and DNA sequences of the virus populations of their mothers. *Proc. Natl. Acad. Sci. USA* **90**, 1721–1725.

Schnittman, S. M., Denning, S. M., Greenhouse, J. J., Justement, J. S., Baseler, M., Kurtzberg, J., Haynes, B. F., and Fauci, A. S. (1990). Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta + and TCR gamma delta+ to human immunodeficiency virus infection: A mechanism for CD4+ (T4) lymphocyte depletion. *Proc. Natl. Acad. Sci. USA* **87**, 7727–7731.

Schubert, U., and Strebel, K. (1994). Differential activities of the human immunodeficiency virus type 1-encoded Vpu protein are regulated by phosphorylation and occur in different cellular compartments. *J. Virol.* **68**, 2260–2271.

Schubert, U., Clouse, K. A., and Strebel, K. (1995). Augmentation of virus secretion by the human immunodeficiency virus type 1 Vpu protein is cell type independent and occurs in cultured human primary macrophages and lymphocytes. *J. Virol.* **69**, 7699–7711.

Schwartz, D. A., and Nahmias, A. J. (1991). Human immunodeficiency virus and the placenta. Current concepts of vertical transmission in relation to other viral agents. *Ann. Clin. Lab. Sci.* **21**, 264–274.

Scott, G. B., Buck, B. E., Leterman, J. G., Bloom, F. L., and Parks, W. P. (1984). Acquired immunodeficiency syndrome in infants. *N. Engl. J. Med.* **310**, 76–81.

Scott, G. B., Fischl, M. A., Klimas, N., Fletcher, M. A., Dickinson, G. M., Levine, R. S., and Parks, W. P. (1985). Mothers of infants with the acquired immunodeficiency syndrome. Evidence for both symptomatic and asymptomatic carriers. *JAMA* **253**, 363–366.

Scott, R. (1994). *Danforths Obstetrics and Gynecology*, 7th edn. JB Lippincott, Philadelphia, Pennsylvania.

Selby, M. J., and Peterlin, B. M. (1990). Trans-activation by HIV-1 Tat via a heterologous RNA binding protein. *Cell* **62**, 769–776.

Semba, R. D. (1997). Overview of the potential role of vitamin A in mother-to-child transmission of HIV-1. *Acta Paediatr. Suppl.* **421**, 107–112.

Semba, R. D., Kumwenda, N., Hoover, D. R., Taha, T. E., Quinn, T. C., Mtimavalye, L., Biggar, R. J., Broadhead, R., Miotti, P. G., Sokoll, L. J., van der Hoeven, L., and Chiphangwi, J. D. (1999). Human immunodeficiency virus load in breast milk, mastitis, and mother-to-child transmission of human immunodeficiency virus type 1. *J. Infect. Dis.* **180**, 93–98.

Shaffer, N., Chuachoowong, R., Mock, P. A., Bhadrakom, C., Siriwasin, W., Young, N. L., Chotpitayasunondh, T., Chearskul, S., Roongpisuthipong, A., Chinayon, P., Karon, J., Mastro, T. D., *et al.* (1999). Short-course zidovudine for perinatal HIV-1 transmission in Bangkok, Thailand: A randomised controlled trial. Bangkok Collaborative Perinatal HIV Transmission Study Group. *Lancet* **353**, 773–780.

Shioda, T., Levy, J. A., and Cheng-Mayer, C. (1991). Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature* **349**, 167–169.

Sleasman, J. W., Aleixo, L. F., Morton, A., Skoda-Smith, S., and Goodenow, M. M. (1996). CD4+ memory T cells are the predominant population of HIV-1-infected lymphocytes in neonates and children. *AIDS* **10**, 1477–1484.

Sova, P., van Ranst, M., Gupta, P., Balachandran, R., Chao, W., Itescu, S., McKinley, G., and Volsky, D. J. (1995). Conservation of an intact human immunodeficiency virus type 1 vif gene *in vitro* and *in vivo*. *J. Virol.* **69**, 2557–2564.

Sprecher, S., Soumenkoff, G., Puissant, F., and Degueldre, M. (1986). Vertical transmission of HIV in 15-week fetus. *Lancet* **2**, 288–289.

Stamatatos, L., and Cheng-Mayer, C. (1993). Evidence that the structural conformation of envelope gp120 affects human immunodeficiency virus type 1 infectivity, host range, and syncytium-forming ability. *J. Virol.* **67**, 5635–5639.

Stanley, S. K., McCune, J. M., Kaneshima, H., Justement, J. S., Sullivan, M., Boone, E., Baseler, M., Adelsberger, J., Bonyhadi, M., Orenstein, J., Fox, C. H., and Fauci, A. S. (1993). Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. *J. Exp. Med.* **178**, 1151–1163.

Strebel, K., Klimkait, T., Maldarelli, F., and Martin, M. A. (1989). Molecular and biochemical analyses of human immunodeficiency virus type 1 vpu protein. *J. Virol.* **63**, 3784–3791.

Strunnikova, N., Ray, S. C., Livingston, R. A., Rubalcaba, E., and Viscidi, R. P. (1995). Convergent evolution within the V3 loop domain of human immunodeficiency virus type 1 in association with disease progression. *J. Virol.* **69**, 7548–7558.

Sundaravaradan, V., Hahn, T., and Ahmad, N. (2005). Conservation of functional domains and limited heterogeneity of HIV-1 reverse transcriptase gene following vertical transmission. *Retrovirology* **2**, 36.

Sundaravaradan, V., Saxena, S. K., Ramakrishnan, R., Yedavalli, V. R., Harris, D. T., and Ahmad, N. (2006). Differential HIV-1 replication in neonatal and adult blood mononuclear cells is influenced at the level of HIV-1 gene expression. *Proc. Natl. Acad. Sci. USA* **103**, 11701–11706.

Tencza, S. B., Miller, M. A., Islam, K., Mietzner, T. A., and Montelaro, R. C. (1995). Effect of amino acid substitutions on calmodulin binding and cytolytic properties of the LLP-1 peptide segment of human immunodeficiency virus type 1 transmembrane protein. *J. Virol.* **69**, 5199–5202.

Tersmette, M., de Goede, R. E., Al, B. J., Winkel, I. N., Gruters, R. A., Cuypers, H. T., Huisman, H. G., and Miedema, F. (1988). Differential syncytium-inducing capacity of human immunodeficiency virus isolates: Frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J. Virol.* **62**, 2026–2032.

Terwilliger, E. F., Cohen, E. A., Lu, Y. C., Sodroski, J. G., and Haseltine, W. A. (1989). Functional role of human immunodeficiency virus type 1 vpu. *Proc. Natl. Acad. Sci. USA* **86**, 5163–5167.

The European Collaborative Study (1994). Caesarean section and risk of vertical transmission of HIV-1 infection. The European Collaborative Study. *Lancet* **343**, 1464–1467.

The European Mode of Delivery Collaboration (1999). Elective caesarean-section versus vaginal delivery in prevention of vertical HIV-1 transmission: A randomised clinical trial. The European Mode of Delivery Collaboration. *Lancet* **353**, 1035–1039.

Thomas, S. L., Oft, M., Jaksche, H., Casari, G., Heger, P., Dobrovnik, M., Bevec, D., and Hauber, J. (1998). Functional analysis of the human immunodeficiency virus type 1 Rev protein oligomerization interface. *J. Virol.* **72**, 2935–2944.

Tiemessen, C. T., and Kuhn, L. (2006). Immune pathogenesis of pediatric HIV-1 infection. *Curr. HIV/AIDS Rep.* **3**, 13–19.

Tiganos, E., Friborg, J., Allain, B., Daniel, N. G., Yao, X. J., and Cohen, E. A. (1998). Structural and functional analysis of the membrane-spanning domain of the human immunodeficiency virus type 1 Vpu protein. *Virology* **251**, 96–107.

Toohey, K., Wehrly, K., Nishio, J., Perryman, S., and Chesebro, B. (1995). Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. *Virology* **213**, 70–79.

Tovo, P. A., de Martino, M., Gabiano, C., Cappello, N., D'Elia, R., Loy, A., Plebani, A., Zuccotti, G. V., Dallacasa, P., Ferraris, G., Palomba, E., and Ruga, E. (1992). Prognostic factors and survival in children with perinatal HIV-1 infection. The Italian Register for HIV Infections in Children. *Lancet* **339**, 1249–1253.

Ullum, H., Lepri, A. C., Victor, J., Skinhoj, P., Phillips, A. N., and Pedersen, B. K. (1997). Increased losses of CD4+CD45RA+ cells in late stages of HIV infection is related to increased risk of death: Evidence from a cohort of 347 HIV-infected individuals. *AIDS* **11**, 1479–1485.

van Dyke, R. B. (1993). Opportunistic infections in pediatric HIV disease. *In* "Pediatric AIDS: Clinical, Pathologic and Basic Science Perspectives" (W. D. Lyman and A. Rubinstein, Eds.), pp. 129–157. New York Academy of Sciences, New York, New York.

Van't Wout, A. B., Kootstra, N. A., Mulder-Kampinga, G. A., Albrecht-van Lent, N., Scherpbier, H. J., Veenstra, J., Boer, K., Coutinho, R. A., Miedema, F., and Schuitemaker, H. (Wout 1994). Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J. Clin. Invest.* **94**, 2060–2067.

Viscarello, R. R., Cullen, M. T., DeGennaro, N. J., and Hobbins, J. C. (1992). Fetal blood sampling in human immunodeficiency virus—seropositive women before elective midtrimester termination of pregnancy. *Am. J. Obstet. Gynecol.* **167**, 1075–1079.

Watts, N. R., Misra, M., Wingfield, P. T., Stahl, S. J., Cheng, N., Trus, B. L., Steven, A. C., and Williams, R. W. (1998). Three-dimensional structure of HIV-1 Rev protein filaments. *J. Struct. Biol.* **121**, 41–52.

Weiblen, B. J., Lee, F. K., Cooper, E. R., Landesman, S. H., McIntosh, K., Harris, J. A., Nesheim, S., Mendez, H., Pelton, S. I., Nahmias, A. J., and Hoff, R. (1990). Early diagnosis of HIV infection in infants by detection of IgA HIV antibodies. *Lancet* **335**, 988–990.

Weinbreck, P., Loustand, V., and Denis, F. (1988). Postnatal transmission of HIV infection. *Lancet* **1**, 482.

Weiss, R. (1993). Cellular receptors and viral glycoproteins involved in retrovirus entry. *In* "The Retroviridae" (J. A. Levy, Ed.), pp. 1–108. Plenum Press, New York.

Welker, R., Harris, M., Cardel, B., and Krausslich, H. G. (1998). Virion incorporation of human immunodeficiency virus type 1 Nef is mediated by a bipartite membrane-targeting signal: Analysis of its role in enhancement of viral infectivity. *J. Virol.* **72**, 8833–8840.

Wellensiek, B. P., Sundaravaradan, V., Ramakrishnan, R., and Ahmad, N. (2006). Molecular characterization of the HIV-1 gag nucleocapsid gene associated with vertical transmission. *Retrovirology* **3**, 21.

Westervelt, P., Trowbridge, D. B., Epstein, L. G., Blumberg, B. M., Li, Y., Hahn, B. H., Shaw, G. M., Price, R. W., and Ratner, L. (1992). Macrophage tropism determinants of human immunodeficiency virus type 1 *in vivo*. *J. Virol.* **66**, 2577–2582.

Wiktor, S. Z., Ekpini, E., Karon, J. M., Nkengasong, J., Maurice, C., Severin, S. T., Roels, T. H., Kouassi, M. K., Lackritz, E. M., Coulibaly, I. M., and Greenberg, A. E. (1999). Short-course oral zidovudine for prevention of mother-to-child transmission of HIV-1 in Abidjan, Cote d'Ivoire: A randomised trial. *Lancet* **353**, 781–785.

Willey, R. L., Theodore, T. S., and Martin, M. A. (1994). Amino acid substitutions in the human immunodeficiency virus type 1 gp120 V3 loop that change viral tropism also alter physical and functional properties of the virion envelope. *J. Virol.* **68**, 4409–4419.

Willey, R. L., Smith, D. H., Lasky, L. A., Theodore, T. S., Earl, P. L., Moss, B., Capon, D. J., and Martin, M. A. (1988). *In vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.* **62**, 139–147.

Wilson, C. C., Brown, R. C., Korber, B. T., Wilkes, B. M., Ruhl, D. J., Sakamoto, D., Kunstman, K., Luzuriaga, K., Hanson, I. C., Widmayer, S. M., Wiznia, A., Clapp, S., *et al.* (1999). Frequent detection of escape from cytotoxic T-lymphocyte recognition in perinatal human immunodeficiency virus (HIV) type 1 transmission: The ariel project for the prevention of transmission of HIV from mother to infant. *J. Virol.* **73**, 3975–3985.

Wolinsky, S. M., Wike, C. M., Korber, B. T., Hutto, C., Parks, W. P., Rosenblum, L. L., Kunstman, K. J., Furtado, M. R., and Munoz, J. L. (1992). Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* **255**, 1134–1137.

Wu, X., Parast, A. B., Richardson, B. A., Nduati, R., John-Stewart, G., Mbori-Ngacha, D., Rainwater, S. M., and Overbaugh, J. (2006). Neutralization escape variants of human immunodeficiency virus type 1 are transmitted from mother to infant. *J. Virol.* **80**, 835–844.

Yang, X., Goncalves, J., and Gabuzda, D. (1996). Phosphorylation of Vif and its role in HIV-1 replication. *J. Biol. Chem.* **271**, 10121–10129.

Yao, X. J., Friborg, J., Checroune, F., Gratton, S., Boisvert, F., Sekaly, R. P., and Cohen, E. A. (1995). Degradation of CD4 induced by human immunodeficiency virus type 1 Vpu protein: A predicted alpha-helix structure in the proximal cytoplasmic region of CD4 contributes to Vpu sensitivity. *Virology* **209**, 615–623.

Yedavalli, V. R., and Ahmad, N. (2001). Low conservation of functional domains of HIV type 1 vif and vpr genes in infected mothers correlates with lack of vertical transmission. *AIDS Res. Hum. Retroviruses* **17**, 911–923.

Yedavalli, V. R., Chappey, C., and Ahmad, N. (1998a). Maintenance of an intact human immunodeficiency virus type 1 vpr gene following mother-to-infant transmission. *J. Virol.* **72**, 6937–6943.

Yedavalli, V. R., Chappey, C., Matala, E., and Ahmad, N. (1998b). Conservation of an intact vif gene of human immunodeficiency virus type 1 during maternal-fetal transmission. *J. Virol.* **72**, 1092–1102.

Yedavalli, V. R., Husain, M., Horodner, A., and Ahmad, N. (2001). Molecular characterization of HIV type 1 vpu genes from mothers and infants after perinatal transmission. *AIDS Res. Hum. Retroviruses* **17**, 1089–1098.

Zaitseva, M. B., Lee, S., Rabin, R. L., Tiffany, H. L., Farber, J. M., Peden, K. W., Murphy, P. M., and Golding, H. (1998). CXCR4 and CCR5 on human thymocytes: Biological function and role in HIV-1 infection. *J. Immunol.* **161**, 3103–3113.

Zapp, M. L., Hope, T. J., Parslow, T. G., and Green, M. R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: A dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci. USA* **88**, 7734–7738.

Zhang, H., Hoffmann, F., He, J., He, X., Kankasa, C., West, J. T., Mitchell, C. D., Ruprecht, R. M., Orti, G., and Wood, C. (2006). Characterization of HIV-1 subtype C envelope glycoproteins from perinatally infected children with different courses of disease. *Retrovirology* **3**, 73.

Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261**, 1179–1181.

Ziegler, J. B., Cooper, D. A., Johnson, R. O., and Gold, J. (1985). Postnatal transmission of AIDS-associated retrovirus from mother to infant. *Lancet* **1**, 896–898.

Zuber, G., McDermott, J., Karanjia, S., Zhao, W., Schmid, M. F., and Barklis, E. (2000). Assembly of retrovirus capsid-nucleocapsid proteins in the presence of membranes or RNA. *J. Virol.* **74**, 7431–7441.

Peter C. Angeletti, Luwen Zhang, and Charles Wood

Nebraska Center for Virology, School of Biological Sciences,
University of Nebraska–Lincoln, Lincoln,
Nebraska 68588

The Viral Etiology of AIDS-Associated Malignancies

I. Chapter Overview

The first documented cases of acquired immunodeficiency syndrome (AIDS) were characterized by the presence of rare Kaposi's sarcoma (KS) skin lesions. More than 10 years later, it was discovered that the causative agent of KS was a γ-herpesvirus, human herpesvirus-8 (HHV-8) (KS-associated herpesvirus, KSHV). It is now abundantly clear that cancers induced by viral agents [such as, Epstein–Barr virus (EBV) and human papillomavirus (HPV)] are exacerbated by human immunodeficiency virus (HIV) infection and subsequent immune suppression. For example, the incidence of primary central nervous system (CNS) lymphoma (PCNSL), Hodgkin's and

Advances in Pharmacology, Volume 56

1054-3589/08 $35.00
DOI: 10.1016/S1054-3589(07)56016-3

high grade B-cell non-Hodgkin's lymphomas (NHL), anal, penile, oral, and invasive cervical carcinomas are much higher in AIDS patients. Also common in the AIDS-afflicted, are hematopoietic cancers, B- and T-cell lymphomas, myelosarcomas, lung cancers, and gastrointestinal tract cancers. The development of highly active antiretroviral therapy (HAART) has proven effective in inducing regression of PCNSL, NHL, KS, and other cancers caused by viruses, extending the life span and quality of life of AIDS patients. However, the general availability of HAART and other antiretrovirals in developing countries, where most HIV infections are reported, is still poor. Furthermore, several reports indicate that HAART is not effective in reversing HPV-induced cervical cancers, for unknown reasons. The development of the prophylactic HPV vaccine offers some hope that future generations can be protected against cervical and penile cancers. However, in countries with high rates of cervical cancers, such as in sub-Saharan Africa, the rate of HIV-positivity approaches 30%, antiretrovirals are scarce, and the HPV vaccine is not available, nor would it be effective for those already infected with HPVs. Thus, better methods of surveillance and management of these malignancies in HIV-positive individuals continues to be a need.

II. Introduction

Since the first report of AIDS over 25 years ago, it was noted that there is a close association between HIV infection and the development of a number of cancers (Levine, 2006; Noy, 2006; Pantanowitz *et al.*, 2006; Wood and Harrington, 2005). They include KS, Hodgkin's and high-grade B-cell NHL, anal, and invasive cervical carcinomas. In fact, AIDS was recognized in 1981 through an unusual increase in the number of cases of KS found among the young adult male having sex with male (MSM) population (Durack, 1981). Since then, increasing numbers of KS as well as other cancers were found in this population. Subsequently in 1982, the US Center for Disease Control and Prevention (CDC) included two malignancies, KS and PCNSL, as definitions for AIDS. A third AIDS-associated malignancy, NHL, was also included as one of the AIDS-defining illnesses in 1987, and a fourth, the invasive cervical carcinoma was added in 1992. In addition to these four malignancies commonly found in AIDS patients, a number of other cancers have also increased substantially in the HIV-1-infected immunosuppressed population. These cancers include multiple myelomas, leukemia, and leiomyosarcomas in children, oral cavity cancers, lung cancers, and Hodgkin's disease (Goedert *et al.*, 1998; Grulich *et al.*, 2002). Prior to the era of HAART, it has been estimated that up to 25% of all cancers in males under 45 years of age in the United States were associated with HIV and up to 30% of all HIV-infected individuals will develop cancer. Especially

with NHL, it has been estimated that individuals with immune deficiency have between 10- and 150-fold higher risk in developing NHL (Biggar and Rabkin, 1996; Grulich and Vajdic, 2005). Moreover, the management and prognosis of these patients were poor, mainly due to the aggressiveness of the tumors on immunosuppression, an increase in hematological toxicity due to treatment, and complications due to opportunistic infections (Kaplan *et al.*, 1997). Treatment outcomes were shown to be poor, regardless of the types of treatment, with the response rate for AIDS-related lymphomas (ARL) of about 50% and the medium survival rate time of between 5 and 8 months (Kaplan *et al.*, 1991; Navarro and Kaplan, 2006).

With the introduction of antiretroviral treatment in the mid-1990s, the spectrum of AIDS-associated malignancies and the epidemiology of the disease has been completely changed. There was a substantial decline in incidence of KS. It has been shown in a Swiss cohort that the standardized incidence rates for KS was 25 for those on HAART versus 239 for those that were not on treatment (Clifford *et al.*, 2005). Similarly, changes in the incidences of ARL were observed for individuals that were on HAART. A number of studies have shown a decrease in incidence and mortality in patients with ARL (Besson *et al.*, 2001; Kirk *et al.*, 2001; Lee and Hurwitz, 2000; Navarro and Kaplan, 2006). The same Swiss cohort study has shown an overall decrease of HNL incidence of about 76% on HAART treatment. Similarly, for PCNSL, the impact of HAART is even more dramatic than other systemic ARL; a combination of radiotherapy with HAART had led to improvement of survival rate (Hoffmann *et al.*, 2001; Newell *et al.*, 2004). HAART alone has also been shown to lead to a regression of PCNSL (McGowan and Shah, 1998). In spite of the effects of HAART on ARL, similar dramatic effects have not been observed with cervical and anal cancers, and the results have been controversial. A study by the Women's Interagency HIV study group has shown an association between HAART and regression of cervical lesions (Minkoff *et al.*, 2001), while other studies did not show such a correlation (Lillo *et al.*, 2001; Schuman *et al.*, 2003). Even with the successes of HAART on a number of AIDS-associated cancers, with the increase of the longevity of treated individuals and the prolonged effects of immunosuppression, it is likely that AIDS-associated malignancies will continue to be a major clinical manifestation of HIV-infected individuals. There is still a lack of widespread antiretroviral therapy in many developing countries where AIDS is epidemic and HIV continues to spread. Better tools for detection and better regimens for management of these malignancies are necessary. Moreover, a better understanding of the biology and the pathogenesis of these cancers is needed.

The mechanisms by which malignancies are induced in HIV-1 infected individuals are not exactly known. It is likely to vary with different types, but a common underlying course is a lack of immunological controls due to immunodeficiencies. In addition, several types of cancers have been linked to

viral etiological agents. In fact, the three major types of cancers included as part of the AIDS-defining illnesses, such as KS, NHL, and cervical cancers, have been linked to infectious viral agents. KS primary effusion lymphomas (PELs) and Castleman's disease have been linked to HHV-8 or KSHV. NHL, PCNSL, Hodgkin's disease, and leiomyosarcoma were found to be associated with EBV. Cervical carcinoma and squamous cell neoplasm were linked to HPV. Substantial information is known about these tumors and their potential etiological agents. A number of mechanisms and viral genes were found to have transforming activities, and they may play a direct and indirect role in tumorigenesis. A better understanding on how infections by these viral agents can lead to tumors will lead to the development of methods to enhance the immune response to control these agents as well as development of therapeutic agents to treat these malignancies. The major focus of this chapter is to examine KSHV-, EBV-, and HPV-associated tumors and what is known about their potential mechanisms in cellular transformation and tumorigenesis.

III. Kaposi's Sarcoma

KS was originally described by Moritz Kaposi in 1872 as an idiopathic, multiply pigmented sarcoma of the skin (Kaposi, 1872). The initiation of a KS lesion is called the *patch* stage of KS, and this stage is characterized by bluish red, well-demarcated, painless maculae, most often unilateral on the lower extremities. The lesion is composed of irregularly shaped vascular spaces present around preexisting vasculature. The lesion progresses into the *plaque* stage as these irregular spaces become lined with endothelial cells and proliferating spindle-shaped cells, presumed to be of endothelial origin. At this stage the lesion appears to be slightly elevated on the skin. The nodular stage can then develop and is characterized by a hard and solid appearance, brownish in color, and can be hyperkeratotic and ulcerative in nature. In advanced stages of KS, lesions are often bilateral and may involve the entire extremity as well as mucosal tissues and present with edema in the surrounding tissue. The nodular lesion is composed of bundles of proliferating spindle-shaped cells, extravasated lymphocytes and erythrocytes in an abundance of slit-like spaces. These lesions become raised on the skin and sometimes coalesce to form large nodular masses (Friedman-Kien and Saltzman, 1990; Iscovich *et al.*, 2000).

There are four epidemiological types of KS recognized: classic, endemic, iatrogenic, and HIV/AIDS-associated KS. Classic KS is the disease originally described by Kaposi (Kaposi, 1872). It predominantly occurs in elderly persons, aged 50–80 years, in the Mediterranean and Eastern European regions or in persons of Jewish ancestry. It is seen primarily in men, with a male:female ratio of 10–15:1. It is infrequently detected in children and

young adults (Friedman-Kien and Saltzman, 1990). Endemic KS was first characterized in African populations from Southern and Central Africa in the early 1960s (Cook, 1962; Lothe and Murray, 1962; Oettle, 1962). Endemic KS was predominantly seen in men, with male:female ratio of 10–17:1, a mean age of 40 years, and presented with multiple, nodular, and sometimes ulcerative lesions in a centrifugal distribution. Health was well maintained while the disease was indolent for several years and rapidly deteriorated as more aggressive lesion growth and dissemination occurred. Iatrogenic- or transplant-associated KS was first recognized in the 1960s and 1970s (Gange and Jones, 1978; Harwood *et al*., 1979; Kapadia and Krause, 1977; Klepp *et al*., 1978; Penn, 1979). It was associated with patients receiving immunosuppressive therapy, most often from organ transplant. KS lesions appeared from 2 months to 8 years after therapy. Iatrogenic KS had a male:female ratio of 2–3:1. The lesions were mostly localized to the skin and infrequently involved the visceral organs and often regressed when therapy was discontinued.

The fourth type of KS is the AIDS-KS. In contrast to the slow development of classic KS, lesions developed rapidly and often disseminated to several locations in the body. AIDS-KS not only involves the lower extremities and skin, but also the upper body, the head regions, and the lymph nodes. It can also disseminate to other organs, such as the spleen, the lungs, the liver, and gastrointestinal track (Hengge *et al*., 2002). AIDS-KS, due to its rapid dissemination, multiple organ involvement, and difficulty with treatment, can be a painful and debilitating disease (Friedman-Kien and Saltzman, 1990). Since the HIV-1/AIDS epidemic, the patterns and clinical manifestations of KS have dramatically changed, especially in Africa. Prior to the early 1980s, Kaposi sarcoma was a rare and indolent tumor of elderly adults, primarily men (Beral, 1991; Friedman-Kien and Saltzman, 1990; Kaposi, 1872). It was extremely rare in children; but with the AIDS epidemic, KS are often seen in young children, and when present, caused a rapidly disseminated disease that failed to respond to chemotherapy and led to death within 1–3 years (Bayley, 1991; Friedman-Kien and Saltzman, 1990).

A. Viral Etiology in KS

With the association of KS to AIDS, an infectious agent has been suspected in the development of KS, including HIV and cytomegalovirus (CMV). A report has shown that herpesvirus-like particles were found in short-term KS tissue culture, and were subsequently identified as CMV (Giraldo *et al*., 1980). However, the involvement of CMV in KS has never been confirmed. In 1994, herpesvirus-like sequences were isolated from biopsy material from an AIDS-KS patient using a subtractive PCR technique called representational difference analysis (Chang *et al*., 1994). This technique allows the preferential amplification of DNA sequences representative

of affected tissue, which is absent in normal tissue from the same individual. The sequence was found to be homologous but not identical to any known herpesviruses. Thus, the virus was named KSHV or HHV-8 since it is the eighth known HHV. KSHV DNA sequences were found only in the KS tissue but not in normal skin tissues. Soon after KSHV DNA sequences were identified, the viral DNA was detected in biopsies from all clinical forms of KS, but was absent in normal tissue (Chang and Moore, 1996; Memar *et al.*, 1995). KSHV is found in all KS lesions, and is mainly located in the vascular endothelial cells and perivascular spindle-shaped cells (Li *et al.*, 1996).

To confirm the etiological role of KSHV in KS, the presence of the virus must be detected in patients prior to the appearance of the disease. Several studies have examined this by detecting viral DNA and seroconversion prior to KS development. In HIV-1-positive patients followed before and after onset of KS, it was observed that patients with KSHV viral DNA at study entry or any time prior to KS were significantly more likely to develop KS than those who were negative for viral DNA prior to KS (Moore *et al.*, 1996c; Whitby *et al.*, 1995). Similarly, in HIV-1-infected patients, those that developed KS were significantly more likely to be KSHV seropositive prior to the onset of KS than those that never developed KS (Gao *et al.*, 1996a; Melbye *et al.*, 1998). However, it is clear that not all KSHV-infected individuals will develop KS, thus KSHV infection plays a major role, but not sufficient, for the development of KS. It is likely that other cofactors, such as immunosuppression, are required for KS development.

In addition to KS, KSHV was found to be associated with two other lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Cesarman *et al.*, 1995; Soulier *et al.*, 1995). PEL is a very rare subtype of NHL, predominantly associated with HIV-infected individuals. PEL was first identified as a subset of body cavity-based lymphomas (BCBL), which were subsequently called PELs (Cesarman *et al.*, 1995). This type of lymphoma is distinguished from others as having a distinctive morphology, bridging large cell immunoblastic lymphoma and anaplastic large cell lymphoma. PELs are extremely rare tumors, and estimated to be about 0.13% of all AIDS-related malignancies in AIDS patients in the United States (Mbulaiteye *et al.*, 2002). PELs are unique as they were found to contain KSHV DNA and are most frequently found in male AIDS patients. Most PELs are coinfected with EBV and KSHV and lack c-myc gene rearrangements, and the role of EBV in this type of tumors will be described in a section. MCD, also known as multicentric angiofollicular lymphoid hyperplasia, is a very rare polyclonal B-cell lymphoproliferative disorder with vascular hyperplasia involving multiple lymphoid organs. This disease is both clinically and morphologically heterogeneous, and is defined using clinical and pathological characteristics (Soulier *et al.*, 1995). Unlike KS, where KSHV sequences can be detected in almost all KS samples, the B cells in MCD are usually not infected. *In situ* hybridization studies showed that

the KSHV-infected cells are mainly located in the mantel zone of the follicle and high levels of the viral homologue of the cellular cytokine vIL-6 can be detected, suggesting that uninfected cells are recruited and stimulated to grow in the affected areas (Katano *et al.*, 2000). It is likely that KSHV may be playing an indirect role in the disease.

B. KSHV Epidemiology

KSHV belongs to the γ-herpesvirus subfamily, which can be further subdivided into two subgroups, gamma-1 or lymphocrytovirus, and gamma-2 or rhadinovirus. EBV is the prototype gamma-1 virus and the simian herpesvirus saimiri is the prototype gamma-2 herpesvirus (Roizmann *et al.*, 1992). KSHV is classified as a gamma-2 rhadinovirus and is the first human virus of this subfamily identified (Moore *et al.*, 1996b). The genome of KSHV is about 165 kb in length, with ~140 kb of long unique DNA surrounded by two terminal repeat regions, 25–35 kb each (Russo *et al.*, 1996). It encodes over 80-open reading frames and has significant homology to the rhadinovirus genus of the γ-herpesvirus subfamily, all of which are known to infect lymphocytes (Moore *et al.*, 1996c). KSHV is the only member of the rhadinovirus genus known to infect humans, but is closely related to another human γ-herpesvirus, EBV, which belongs to the lymphocryptovirus genus. A feature of γ-herpesviruses like KSHV is its ability to incorporate or pirate host genes such as cyclin D and growth factor IL-6 into their genome (Moore *et al.*, 1996a), and these genes can then play a role in the replication, survival, and transformation function of the virus. Deciphering the functions of these viral genes will lead to a better understanding of viral pathogenesis and oncogenesis.

Unlike most other herpesviruses, KSHV infection does not seem to be widely distributed in most populations. The detection of KSHV infection relies on the presence of antibodies against either lytic and/or latent antigens and varies among the different tests that were used in different seroprevalence studies. There are several different methodologies for the detection of antibodies to KSHV. Most of these assays are based on immunofluorescence antigen (IFA), utilizing B-cell lymphoma cell lines known to be infected with KSHV as the antigen source or based on ELISA with recombinant immunogenic proteins or peptides of KSHV. The performance of these assays can be quite variable, and could account for the differences in seroprevalence reported in different studies (Pellett *et al.*, 2003; Rabkin *et al.*, 1998; Tedeschi *et al.*, 2002). In North America and Northern and Western Europe, KSHV seroprevalence in adult general population or blood donors ranges from 0% to 8% (Challine *et al.*, 2001; de Sanjose *et al.*, 2002; Gambus *et al.*, 2001; Gao *et al.*, 1996b; Goedert *et al.*, 1998; Lennette *et al.*, 1996; Simpson *et al.*, 1996). In these countries, the seroprevalence of KSHV in different risk groups mirrors the incidence of AIDS-KS, with a seroprevalence rate of

between 25% and 50% among homosexual men. On the contrary, the reported seroprevalence of KSHV is high in the adult general population in regions of Brazil, French Guiana, the Mediterranean basin, and Central and Southern Africa, where it ranges from 10% to over 80%; these regions are considered endemic for KSHV (Cunha *et al.*, 2005; Freitas *et al.*, 2002; Kazanji *et al.*, 2005; Klaskala *et al.*, 2005; Mayama *et al.*, 1998; Mbulaiteye *et al.*, 2003; Olsen *et al.*, 1998; Plancoulaine *et al.*, 2000, 2004; Wilkinson *et al.*, 1999). Central African countries, like the Republic of Congo, Uganda, and Zambia, also have the highest KSHV infection rates in the world (Gao *et al.*, 1996b). Therefore, KSHV seroprevalence tracks very closely with KS, with the highest infection rate in geographic areas where classic or endemic forms of KS are more common.

C. Viral Oncogenesis

A common property shared between KSHV and several other members of the γ-herpesviruses, such as EBV, is their ability to cause proliferation of infected host cells and lead to neoplasm in the infected host. Infection of primary endothelial cells by KSHV has been shown to lead to morphologic and phenotypic changes of these cells that resemble the characteristics of KS spindle cells, suggesting that KSHV can lead to malignant transformation and plays a role in the pathogenesis of KS (Flore *et al.*, 1998; Foglieni *et al.*, 2005; Moses *et al.*, 1999). KSHV has been shown to encode for a number of viral genes that may contribute to tumorigenesis. These genes include both unique viral genes and gene homologues of cellular genes. Some of these viral genes have transformation potential, such as the viral K1 gene, kaposin and viral G protein-coupled receptor (vGPCR). Others are viral proteins that have homology to their cellular counterparts. These proteins may deregulate cell growth and lead to transformation. These genes include the viral IL-6, viral IL-10, viral CC-class chemokines, and viral FLICE-inhibitory protein (vFLIP). Yet there are other viral genes that are involved in maintaining viral latency such as the latency-associated nuclear antigen (LANA) and K15. These genes are involved in a number of strategies that the virus uses in sustaining viral infection in pathogenesis and in the development of malignancies. These mechanisms involved the stimulation of cell proliferation, activation of cellular gene expression, immune suppression, and modulation of immune surveillance. These viral genes may also participate indirectly via upregulation of other viral genes. A summary of the viral genes involved is shown in Table I.

The KSHV K1 gene is the first open reading frame of the viral genome. It encodes a transmembrane protein with a cytoplasmic domain containing a functional immunoreceptor tyrosine-based activation motif (ITAM) (Lagunoff and Ganem, 1997; Lee *et al.*, 2003). ITAM motifs are known to be involved in signal transduction on ligand–receptor interaction. However,

TABLE I Potential KSHV Genes Involved in Tumorigenesis

Viral gene	*Function*
K1 (KSHV open reading frame 1)	Signal transduction on receptor binding. Homologue of the herpesvirus saimiri STP transforming gene and is involved in the deregulation of NF-κB
K12 (Kaposin)	Two forms, Kaposin A and B. Kaposin A is a type II membrane protein; Kaposin B is involved in the MAPK signaling pathway
vGPCR (viral G protein-coupled receptor)	Homologue of cellular IL-8 receptor and binds to CXC and CC chemokines. It stimulates MAPK pathway and leads to secretion of VEGF
vIL-6 (viral IL-6)	Homologue of cellular IL-6, it supports cell growth and protects the cells from undergoing apoptosis
K4, K4.1, and K6 (KSHV open reading frame 4, 4.1, and 6)	Viral homologues of cellular CC chemokines such as RANTES and MIP-1α. They induce signal transduction and enhance angiogenesis
vFLIP (viral FLICE-inhibitory protein or ORF71)	Homologue of cellular FLIP, it activates NF-κB pathway and protects cells from apoptosis
LANA (viral latency associated nuclear antigen)	Important in the maintenance of viral latency and binds to viral genome and a number of cellular factors, such as p53

K1 signaling appears to be constitutive and may be responsible for the activation and proliferation of infected B lymphocytes by inducing phosphorylation of several cellular signal transduction proteins (Lagunoff *et al.*, 1999; Lee *et al.*, 1998a,b). The K1 gene was shown to have transforming activities; it transformed mouse cells *in vitro* and caused tumors in nude mice (Lee *et al.*, 1998b). K1 was also found to be able to replace the transforming gene (STP) of the herpesvirus saimiri and caused immortalization of marmoset T lymphocytes (Lee *et al.*, 1998b). Transgenic mice expressing K1 gene developed malignant plasmacytomas and these cells have elevated levels of NF-κB and other cellular transcription factors, further suggesting that deregulation of normal cellular functions by K1 may lead to the development of B-cell lymphomas (Prakash *et al.*, 2002).

The viral kaposin or open reading frame K-12 has also been found to play a role in transformation. The kaposin gene is expressed during latency but can also be induced on lytic replication (Muralidhar *et al.*, 1998, 2000; Sadler *et al.*, 1999; Wang and Boshoff, 2005). This gene is most abundantly expressed during latency and has a complex translational pattern resulting in three different forms of the kaposin proteins, known as A, B, and C (Sadler *et al.*, 1999). Kaposin A is a type II membrane protein, and it was shown to have transforming activities and can transform cells *in vitro*; the transformed cells caused tumors in nude mice (Kliche *et al.*, 2001; Muralidhar *et al.*, 1998, 2000). Its transforming activities were linked to its interaction with a guanine nucleotide exchange factor for ADP-ribosylation factor (ARF) GTP hydrolase (GTPase) known as cytokesin-1 and a domain known as the LXXLL motif on the protein seems to be important for transformation (Tomkowicz *et al.*, 2005). Kaposin B appears to play a role in cytokine release; it binds to host cell protein kinase, such as mitogen-activated protein kinase (MAPK)-associated protein kinase 2, which plays an important role in the proinflammatory p28 MAPK signaling pathway, resulting in an enhancement of inflammatory cytokine secretions to enhance the development of KS (McCormick and Ganem, 2006). Currently, nothing is known about the function of Kaposin C.

The viral GPCR is a lytic viral gene and is a homologue of the cellular IL-8 receptor except that it is constitutively expressed. It binds to the cysteine-x-cysteine (CXC) and cysteine–cysteine (CC) families of chemokines (Arvanitakis *et al.*, 1997; Cesarman *et al.*, 1996; Gershengorn *et al.*, 1998). KSHV GPCR has been shown to transform cell *in vitro* and promote immortalization of endothelial cells and tumor formation in the presence of KSHV latent genes, suggesting that both lytic and latent genes are important during the development of KS (Arvanitakis *et al.*, 1997; Bais *et al.*, 2003). The signal transduction property of GPCR is important for its transforming activities. It is known to stimulate the MAPK and PI3K pathways, leading to the stimulation of a large number of cellular genes that could enhance

the proliferation of KSHV-infected cells. Activation of vGPCR has been associated with an increase in the secretion of vascular endothelial cell growth factors (VEGF) and VEGF receptors, which leads to an induction of the angiogenic response, to enhance the growth of immortalized KSHV-infected cells both *in vitro* and *in vivo* through a paracrine pathway mediated by vGPCR (Montaner *et al*., 2003; Sodhi *et al*., 2000; Yang *et al*., 2000).

In addition of vGPCR, there are other viral genes that have homology to cellular homologue genes, such as the viral IL-6, viral CC-chemokines (vCCLs), and vFLIP. KSHV vIL-6 has both sequence and functional homology to the cellular IL-6, but they differ in their ability to bind to cellular receptors. Cellular IL-6 requires both the α and the gp130 subunits for binding and signaling, whereas vIL-6 requires only the gp130 subunit (Molden *et al*., 1997). KSHV-infected PEL MCDD cells secrete vIL-6 to support the growth of the infected cells and also protect the cells from the antiviral effects mediated by the interferon pathway (Moore *et al*., 1996a; Nicholas *et al*., 1997). Thus, vIL-6 appears not only to have the ability to support the growth of infected cells, but also can protect infected cells from the effects of interferon. In addition to vIL-6, several viral genes, K4, K4.1, and K6, encode viral homologues of the cellular CC chemokines, such as RANTES and MIP-1α (Boshoff *et al*., 1997). These chemokines can induce signaling transduction, enhance angiogenesis, and contribute to the tumorigenesis process (Nakano *et al*., 2003). The KSHV FLIP gene ORF71 is a latent viral gene which encodes the FLIP protein and is structurally most homologous to the cellular FLIP. It facilitates lymphoma cell growth by activating NF-κB expression and its signaling pathway, and by conferring the infected cells resistance to apoptosis (Djerbi *et al*., 1999; Guasparri *et al*., 2004; Thome *et al*., 1997). In addition, vFLIP was shown to induce morphological changes in primary endothelial cells to become spindle-like in shape, similar to KS tumor cells (Grossmann *et al*., 2006). These together with the antiapoptotic functions of vFLIP reflect two features that are known to be the hallmark of KS.

Another KSHV latently expressed protein that may contribute to neoplasm is LANA. The KSHV LANA is a nuclear phosphoprotein that is important for the maintenance of viral latency (Dittmer *et al*., 1998). It has been shown to bind to the terminal repeat region of the viral genome and tethered the viral episome to the host chromosome so that it can be maintained during cellular mitotic replication and segregation (Ballestas *et al*., 1999; Cotter *et al*., 2001). LANA is a multifunctional viral protein and can bind to a number of cellular proteins. It can bind to tumor suppressors p53 and Rb and protect infected cells from apoptosis (Friborg *et al*., 1999; Radkov *et al*., 2000). This together with its ability to upregulate β-catenin expression promotes S-phase entry, modulates cell cycle pathways, and contributes to the development of neoplasm (Fujimuro *et al*., 2003).

IV. AIDS-Associated Lymphomas

As persons with HIV infection survive longer despite significant immunosuppression, more cases of malignancy are likely to appear. Although HIV infects T lymphocytes, AIDS-associated lymphomas are of B lymphoid origin in at least 95% of all cases described. As with other lymphomas, AIDS-associated lymphomas also fall into two broad categories: AIDS-associated Hodgkin's disease and NHL. AIDS-associated NHLs are primarily encountered in patients with more advanced HIV infection, with a low CD4 count. Although Hodgkin's disease is included in the HIV-associated lymphomas in the WHO classification, it will not be discussed in this chapter because the relation between HIV infection, AIDS, and Hodgkin's disease is unclear (Carbone and Gloghini, 2005). Whether HIV infection promotes the development of Hodgkin's disease or merely modifies its clinical progression is not yet known.

Mechanistic studies have revealed that potential factors contributing to lymphoma development. Three major factors promoting the development of lymphoma are HIV-induced immunosuppression, chronic antigenic stimulation, and cytokine overproduction. These alterations are associated with the development of oligoclonal B-cell expansions. The appearance of lymphomas is characterized by the presence of a monoclonal B-cell population displaying a variety of genetic lesions, including EBV infection, c-myc gene rearrangement, bcl-6 gene rearrangement, ras gene mutations, and p53 mutations/deletions. The number and type of genetic lesions varies according to the anatomic site and histopathology. Thus, it is apparent that more than one pathogenic mechanism is operational in the development and progression of AIDS-associated lymphomas (Carbone and Gloghini, 2005; Epeldegui *et al.*, 2006). This chapter attempts to summarize the potential role of viral etiological factors, especially EBV, on the development of the malignancies.

A. EBV, Its Latency, and Its Role in AIDS-Associated Lymphomas

EBV is a ubiquitous human-herpesvirus that infects about 95% of the adult population worldwide. The majority of primary infections occur in early childhood and are generally asymptomatic. However, when primary infection is delayed until adolescence or adulthood, as often occurs in developed countries, it may cause infectious mononucleosis (IM), which is a self-limiting lymphoproliferative disorder characterized by increased numbers of EBV-infected B cells in peripheral blood and massive oligoclonal expansion of EBV-specific $CD8^+$ T cells.

The biologic hallmark of the EBV–cell interaction is latency. EBV establishes several latencies on infection of target cells. Three types of latency

have been described, each having its own distinct pattern of EBV gene expression. Type I latency is exemplified by Burkitt's lymphoma (BL) tumors *in vivo* and earlier passages of cultured cell lines derived from BL biopsies. Epstein–Barr Nuclear Antigen 1 (EBNA-1) and small EBV-encoded, nonpolyadenylated nuclear RNAs (EBER-1 and -2) are expressed in this form of latency. Type II latency is exemplified by NPC and Hodgkin's disease. EBNA-1, latent membrane protein 1 (LMP-1), LMP2A, and LMP2B proteins, as well as EBERs, are expressed in type II latency. EBV transforms adult primary B cells into continually growing lymphoblastoid cell lines (LCLs) and concomitantly establishes type III latency *in vitro*. Nine viral proteins are expressed, including six nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP) and three integral membrane proteins (LMP-1, LMP-2A, and LMP-2B) plus EBERs (Kieff, 1996; Rickinson and Kieff, 1996). Extensive mechanistic studies on EBV transformation have identified several key viral genes that contribute to the viral transformation processes. They are LMP-1, LMP-2, EBNA-1, and EBNA-2.

B. Latent Membrane Protein 1 (LMP-1)

The role of LMP-1 in EBV transformation of primary B lymphocytes is well established. LMP-1 was initially identified as a viral oncoprotein on the basis of its ability to transform rodent cells. Fibroblasts constitutively expressing LMP-1 demonstrate reduced serum requirements, increased growth in soft agar, loss of contact inhibition, and tumorigenic potential in nude mice (Dawson *et al.*, 1990; Fahraeus *et al.*, 1990; Wang *et al.*, 1985). Moreover, expression of LMP-1 as a transgene in mice under the control of the immunoglobulin promoter/enhancer results in increased frequency of B-cell lymphomas, indicating that this viral protein has oncogenic properties *in vivo* (Kulwichit Raab-Traub). In viral transformation assays with primary B cells, deletion of LMP-1 prevents the transformation of primary B cells (Izumi *et al.*, 1997; Kaye *et al.*, 1993), and inhibition of LMP-1 expression in EBV-transformed cells reverts the transformed phenotypes (Kilger *et al.*, 1998). Thus, LMP-1 is required for EBV transformation of primary B cells *in vitro* in tissue culture system.

LMP-1 expression alone modulates cellular gene expression that is responsible for phenotypic and functional changes associated with EBV latency. These changes include the upregulation of adhesion molecules (LFA-1, ICAM-1, and LFA-3), B-cell activation markers (CD23, CD30, CD40, and CD71), transcriptional factors [signal transducer and activator of transcription 1 (STAT-1), -2, IRF-7], and antiapoptotic genes (Bcl-2, BclxL, Mcl1, and A20). Thus, LMP-1 appears to be a central effector of altered cell growth, survival, adhesive, invasive, and even antiviral potential in EBV-infected cells (Fries *et al.*, 1996; Miller *et al.*, 1995; Wang *et al.*, 1985, 1990; Yoshizaki *et al.*, 1998).

Extensive studies have led to some insight into the molecular mechanisms underlying the function of LMP-1. LMP-1 is an integral membrane protein with six transmembrane-spanning domains and a long C-terminal domain located in the cytoplasm (Kieff, 1996; Liebowitz *et al.*, 1986). LMP-1 acts as a constitutively active, receptor-like molecule that does not need the binding of a ligand (Gires *et al.*, 1997). The six transmembrane domains mediate oligomerization of LMP-1 molecules in the plasma membrane, a prerequisite for LMP-1 function (Floettmann and Rowe, 1997; Gires *et al.*, 1997). Two regions in the C-terminus of LMP-1 have been shown to initiate signaling processes, the C-terminal activator regions 1 (CTAR-1, amino acids 194–231) and 2 (CTAR-2, amino acids 332–386) (Huen *et al.*, 1995; Mitchell and Sugden, 1995). In a more refined analysis, several kinds of functional domains have been identified. The PXQXT domain is located within the CTAR-1 and is involved in the interaction with tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), and the binding of TRAFs to LMP-1 results in the induction of the NF-κB and AP-1 transcription factors. It is thus apparent that LMP-1 shares functional properties with members of the TNF-receptor superfamily, particularly CD40. Moreover, LMP-1 can partially restore the wild-type phenotype of mice deficient in CD40 (Devergne *et al.*, 1996, 1998; Miller *et al.*, 1997, 1998; Sandberg *et al.*, 1997). LMP-1 also interacts with TNFR-associated death domain protein (TRADD) and receptor-interacting protein (RIP) at the C terminal (Devergne *et al.*, 1998; Floettmann and Rowe, 1997; Izumi *et al.*, 1997, 1999; Izumi and Kieff, 1997; Kaye *et al.*, 1996). Interaction with these two molecules contributes the majority of the NF-κB activity induced by LMP-1. Also, c-Jun N-terminal kinase (JNK) is activated by CTAR-2. The domain for activation is mapped to most C-terminal amino acids and apparently overlaps the TRADD interaction domain. However, whether TRADD and TRAF2 are involved in the activation of JNK is disputed (Eliopoulos and Young, 1998; Eliopoulos *et al.*, 1999; Kilger *et al.*, 1998). In addition, two janus kinase 3 (JAK3) binding sites have been identified between CTAR-1 and CTAR-2. JAK3 binding to the sites is responsible for the activation of STAT-1 (Gires *et al.*, 1999). However, some other experimental evidence suggests an alternative mechanism (Brennan *et al.*, 2001; Higuchi *et al.*, 2002; Zhang *et al.*, 2004b). In summary, the hijacking of these cellular signaling pathways by LMP-1 is likely to contribute to the pathogenesis of most EBV-associated disorders through the simultaneous or sequential activation of signals involved in the promotion of cell activation, growth, and survival.

C. Latent Membrane Protein 2

The LMP-2 protein contains multiple membrane spanning domains and cytoplasmic N- and C-terminal domains and forms aggregates in the membrane of EBV-infected B cells. The N-terminal domain can bind to the tyrosine kinases Lyn and Syk through their SH2 domains (Longnecker *et al.*, 2000).

These kinases are recruited to the B-cell receptor (BCR) following antigen cross-linking, and their subsequent activation stimulates downstream events resulting in B-cell differentiation and proliferation. LMP-2A may work as a decoy protein sequestering Lyn and Syk to inhibit BCR signaling, which make LMP-2 an inhibitor of EBV lytic replication induced by BCR ligation (Longnecker, 2000; Longnecker *et al.*, 2000). The property of LMP-2 may play a major role in mediating EBV persistence *in vivo*.

Unlike LMP-1 and EBNA-2, the LMP-2 protein is not essential for B-cell transformation *in vitro*. Nevertheless, the constant expression of LMP-2 in EBV-carrying memory B cells from healthy individuals suggests that LMP-2 probably plays an important role in viral persistence. LMP-2 in transgenic mice model has shown that LMP-2 provides survival signals that allow immature B cells to progress through developmental checkpoints and prevent cell death. This property may be related to the ability of LMP-2A to activate the serine-threonine kinase Akt. Akt is a multifunctional mediator of phosphatidylinositol 3-kinase (PI3-K) activity. Activation of the pathway results in the constitutive delivery of an antiapoptotic signal. Akt is also involved in the control of B-cell proliferation because chemical inhibition of PI3-K induced growth arrest of EBV-transformed B cells (Brennan *et al.*, 2002).

D. Epstein–Barr Nuclear Antigen1 (EBNA-1)

The EBNA-1 protein is expressed in all EBV latency states and all EBV-associated tumors. The only exception is that EBNA-1 is hardly detectable in the circulating EBV-infected memory B cells. This alone suggests that the biologic properties of this protein are critical for EBV-mediated transformation (Kieff, 1996). EBV establishes itself efficiently in infected B lymphocytes, where it exists as 165 kb, circular episome which is duplicated once per cell cycle. Remarkably only EBNA-1 protein is required for the synthesis and partitioning of the viral episomes. EBNA-1 binds to two regions of the viral origin of replication (OriP), referred to as the family of repeats (FR) and the dyad symmetry (DS) element. FR is essential for episome maintenance, while DS is required for initiation of OriP-dependent DNA replication. EBNA-1 is also a transcriptional regulator that modulates the activity of the viral promoters: Wp and Cp and its own latent promoter Qp. Moreover, EBNA-1 is essential to drive transcription of EBV's transforming genes after infection of primary B lymphocytes (Altmann *et al.*, 2006). In addition, EBNA-1 can inhibit apoptosis in B cells that likely contributes to the persistence of EBV-infected cells and survival of EBV-transformed cells *in vivo*.

E. EBNA-2

The EBNA-2 protein is localized in the nucleus and is one of the first viral proteins expressed during EBV infection of primary B lymphocytes.

In cooperation with EBNA-LP (also known as EBNA-5), EBNA-2 induces the transition of resting B cells from G0 to G1. EBNA-2 is a key regulator of viral gene expression, being able to stimulate transcription from the major latency BamHI-C promoter, which directs expression of all the EBNA genes, and the promoters of LMP-1 and LMP-2. In addition, EBNA-2 modulates the transcriptional activity of some cellular genes. Cellular C-fgr, c-myc, CD21, CD23, and EBI1/BLR2 are upregulated whereas the immunoglobulin heavy chain genes are repressed in lymphocytes. There is no evidence that EBNA-2 binds to DNA directly. Rather, its transcriptional activity is mainly mediated by its interaction with the DNA-binding cellular protein RBP-Jκ (also called RBP-J, CBF1, KBF2, or CSL). EBNA-2 is essential for EBV-induced immortalization of B lymphocytes and complex formation with RBP-Jκ is crucial for such activity. RBP-Jκ is expressed ubiquitously and is an important component of the Notch signaling pathway that is involved in the regulation of lymphoid development. Notch proteins are a family of transmembrane receptors that on ligand binding undergo proteolytic cleavage of their intracellular domain (Notch1 IC). The cleaved and released Notch1 IC fragment is transported to the nucleus where it interacts with RBP-Jκ and modulates the activity of target promoters. Although Notch1 IC and ENBA-2 share the ability to transactivate genes by interacting with RBP-Jκ, the set of promoters regulated by Notch1 IC and EBNA-2 is overlapping but not identical.

Generally, transformation of a cell requires multiple molecular events (Cole and McMahon, 1999; Kelekar and Cole, 1986; Kohl and Ruley, 1987; Ralston, 1991; Shalloway *et al*., 1987; Weinberg, 1985, 1989). Several viral genes, such as LMP-1 and EBNA-2, are required for the transformation of primary B cells *in vitro* and are believed to drive EBV transformation process *in vivo*. EBV contributes to the cellular transformation processes through the activity of viral proteins that act cooperatively to modify cellular gene expression that involved in cell proliferation, apoptosis, angiogenesis, immune regulation, and signal transduction (Cahir-McFarland *et al*., 2000; Chen *et al*., 2003; Fries *et al*., 1996; Henderson *et al*., 1991; Miller *et al*., 1995; Wang *et al*., 1985, 1990; Yoshizaki *et al*., 1998; Zhang and Pagano, 1999; Zhang *et al*., 2004a,c).

V. AIDS-Associated NHL

AIDS-associated NHL is generally divided into three subtypes: PCNSL, PEL ("body cavity"), and systemic NHL (Knowles, 2003). The vast majority of AIDS-associated NHL is clinically aggressive B-cell-derived neoplasms. Approximately 80% arise systemically (nodal and/or extranodal), and the remaining 15–20% arise as PCNSL. A small proportion is BCBLs (Knowles, 2003). EBV apparently contributes to the development of these tumors in various fashions.

A. Primary Central Nervous System Lymphoma

PCNSL is a form of NHL arising within and confined to the CNS. It was first described by Bailey in 1929 as a perithelial sarcoma (Bailey, 1929). Subsequent classifications have included reticulum cell sarcoma and microglioma. Improvements in histopathology and immunohistochemical techniques definitively established the lymphoid nature of PCNSL. PCNSL accounts for up to 15% of NHLs in HIV-infected patients compared to only 1% of NHLs in the general population. The reported incidence of PCNSL in HIV-infected patients is 2–6% (at least 1000 times higher than in the general population) and has been as high as 10% in autopsy series. Although CNS involvement also occurs in AIDS-associated systemic lymphoma in the form of secondary spread of the tumor to the meninges, the disease is limited to the CNS in PCNSL (Cheung, 2004; Cingolani *et al.*, 2005; Eichler and Batchelor, 2006; Gates and Kaplan, 2002; Sparano, 2003). Prior to the introduction of HAART, the incidence of PCNSL in the HIV-infected population was continuing to rise. However, the impact of these new drug regimens on the CD4 count may result in a decline in PCNSL, as the susceptibility to PCNSL is inversely proportional to the CD4 count (Sparano *et al.*, 1999). In normal individuals, a small number of circulating B cells enter the CNS, and may do so in increased numbers as HIV infection advances (Cingolani *et al.*, 2005; Ivers *et al.*, 2004). EBV establishes latent, life-long infection in over 90% of adults. During the course of HIV infection, EBV-specific T cells progressively lose the capacity to produce interferon-gamma in response to EBV peptides. In addition, EBV-positive B lymphocytes occur more frequently in the CNS of HIV-infected individuals than in normal brains, which may set up a stage for EBV transformation of these infected cells.

EBV appears to play a major pathogenetic role in AIDS-associated PCNSL: (1) EBV genome within tumors is present in more than 95% of AIDS patients, but in only 0–20% (probably <5%) of immunocompetent patients. (2) More than half of AIDS PCNSL examined so far expressed at least EBNA-2, LMPs, and EBERs, a pattern referred to as type III latency and closely resembling that seen in transformation of primary B lymphocytes *in vitro* EBV (Cingolani *et al.*, 2005; Ivers *et al.*, 2004). Expression of type III latency genes leads to a variety of cellular effects, including upregulation of the genes that are involved in transformation, such Bcl-2 and IRF-7, and inactivation of the p53 and Rb tumor suppressor gene products. It is believed that the EBV triggers certain PCNSL *in vivo* in a process similar to transformation processes of primary B cells *in vitro* (Pagano, 1999).

B. Primary Effusion Lymphoma

PELs, also known as BCBL, are B-cell NHLs and most frequently occur in AIDS patients as lymphomatous effusions in the serous cavities without a

detectable solid tumor mass. In the setting of AIDS, the clinical course for most of these lymphomas is extremely aggressive, with a mean survival from diagnosis of 5–7 months (Nador *et al.*, 1996). While PELs are almost universally KSHV positive, the majority of PELs have concomitant EBV infection (reviewed in Dourmishev *et al.*, 2003; Drexler *et al.*, 1998; Moore and Chang, 2001). EBV apparently establishes type II latency in PELs with low levels of LMP-1 expression (Callahan *et al.*, 1999; Fassone *et al.*, 2000; Horenstein *et al.*, 1997; Lacoste *et al.*, 2000).

Both KSHV and EBV are oncogenic herpesviruses. It is thus interesting to examine if there are any interactions between the two viruses in PELs. Comparing to KSHV-only PELs, coinfection with EBV enhances the tumorigenecity of the dually infected PELs in severe combined immunodeficiency (SCID) mice model (Trivedi *et al.*, 2004). The mechanism of the enhancement is currently unknown. However, LMP-1 might be involved in the enhancement because LMP-1 is expressed and its expression may be enhanced by both KSHV latent gene (LANA) and lytic gene (K-RTA). Although expression of LMP-1 at least in some of the PEL specimens strongly suggests the contribution of EBV to the development of the tumor, this enhancement was not apparent in clinical settings, possibly due to the fact that patients with PEL are usually at advanced HIV-infection stage.

At molecular levels, unique sets of cellular genes are expressed in dually infected, but not singly KSHV-infected PELs (Fan *et al.*, 2005). KSHV reduces the expression of EBV EBNA-1 and represses EBV EBNA-2 activation (Krithivas *et al.*, 2000). EBV inhibits KSHV lytic replication, in part, because of a regulatory loop in which KSHV lytic gene induces EBV LMP-1, and LMP-1, in turn, inhibits the lytic gene expression programs of KSHV (Xu *et al.*, 2007). Like EBV EBNA-2, KSHV replication and transcriptional activator (K-RTA) bind to RBP-Jκ (Liang *et al.*, 2002), a key cellular target of the EBV latent transforming program (Zimber-Strobl and Strobl, 2001). Also, KSHV induces the expression of CD21, the cellular receptor for EBV, and thus facilitates EBV infection (Chang *et al.*, 2005). All this data suggests that coordinated cellular transformation by the two viruses is a possibility. However, how these two viruses interact and affect each other and the pathobiology of PELs remains to be determined.

C. Systemic AIDS-Associated NHL

Systemic AIDS-associated NHLs are aggressive B-cell lymphomas of high or intermediate grade and heterogeneous in nature. Approximately one-third can be classified as small noncleaved cell lymphomas, which are Burkitt or Burkitt-like lymphomas. The remaining two-thirds of the lymphomas are diffuse large cell lymphomas, which are immunoblastic lymphomas or large noncleaved cell lymphomas (Brockmeyer and Barthel, 1998).

EBV infection and c-myc oncogene rearrangements are the two well-established factors in the pathogenesis of the systemic NHL. The diffuse large cell lymphomas frequently express EBV latency type III antigens including EBNA-2 and LMP-1 and -2, which have transforming activity *in vitro* are well established. EBV establishes type I latency expressing only EBNA-1 in BLs. However, the EBV genome can be detected in only 60% of the diffuse large cell lymphomas, and in around 30% of the AIDS-associated BLs. Because EBV is less frequently detected in systemic lymphomas, and the increasing incidence of this type of cancer in HIV-infected patients, some additional common latent or chronic viral infections may be involved in the development of these tumors (Mueller, 1999; Shibata *et al*., 1993). In the setting of underlying HIV infection, systemic NHL truly behaves as an opportunistic neoplasm, overwhelming those immune mechanisms that may normally attempt to keep the cancer in check.

VI. HPV-Associated Cancers

A. Types of HPV-Induced Cancers

HPVs infect the stratified epithelia of skin or mucosa, where they cause benign warts. Of the 200 different types of HPVs (Cates and Dallabetta, 1999), the most common HPVs (types 2 and 4) are those that cause warts on the hands and feet of affected individuals (Howley, 1996). Anogenital tract HPVs, of which ~40 have been identified, are divided into those which confer a "low risk" (types 6, 11, and 42) or a "high risk" (types 16, 18, and 31) for cervical cancer (Howley, 1996; Sakai *et al*., 1996; zur Hausen, 1999, 2000). Studies performed by Harold Zur Hausen's laboratory provided the first definitive evidence that HPVs were present in genital cancers (Bosch *et al*., 1991; Durst *et al*., 1983; Gissmann *et al*., 1984; Schwarz *et al*., 1985; zur Hausen, 1999, 2000; zur Hausen *et al*., 1975, 1981). After more than 20 years of work, HPVs are now recognized as a necessary cause in 95% of invasive cervical cancers worldwide (Walboomers *et al*., 1999). Approximately 20 million US adults are infected with genital HPVs and there are 5.5 million new infections each year, representing a major public health concern (Cates and Dallabetta, 1999). In human cervical cancer cells, high-risk papillomavirus DNA is most often found integrated into the host chromosomes (Londesborough *et al*., 1996; Schwarz *et al*., 1985; Yee *et al*., 1985b).

HPVs are most commonly associated with cervical cancer, although, it is now known that many cancers are induced by HPV, including penile, anal, oral, and conjunctival cancers (Durst *et al*., 1983; Koutsky, 1997; Minchiotti *et al*., 2006; Newton *et al*., 2002; Syrjanen, 2003; Waddell *et al*., 1996). High-risk HPVs have also been implicated recently in ~30% of oral cancers (Gillison *et al*., 2000). In fact, HPVs are responsible for cancers in the

tonsils, the palate, gums, tongue, and the larynx (Aaltonen *et al.*, 2005; de Villiers *et al.*, 1986; El-Mofty and Lu, 2003; Lopez Amado *et al.*, 1996; Milde-Langosch *et al.*, 1989; Mineta *et al.*, 1998; Sinclair *et al.*, 2005; Syrjanen, 2005; Yoshpe, 1995). High-risk HPVs have been further implicated in upper respiratory tract and lung cancers (Cheah and Looi, 1998; Clarke *et al.*, 1991; de Villiers *et al.*, 1986). Furthermore, evidence suggests that some digestive cancers are also HPV positive (Milde-Langosch *et al.*, 1989; Nakano, 1994). HPVs are some of the most ubiquitous and stable viruses in nature, thus, it is not surprising that multiple tissues are targets of HPV-induced tumorigenesis.

Penile cancers are much less common than cancers of the cervix, for reasons that are not entirely clear (Gloeckler Ries *et al.*, 2003). Despite the fact that men seldom show clinical signs, it is likely that many could be persistently infected and that the progression to penile cancer occurs under immunosuppressive conditions. Cancers of the vulva and vagina are also relatively rare compared to cervical cancers. The reason for these differences in incidence of cancers in different tissues is related to the cell-type infected. The cells of the cervical transformation zone at the squamous and columnar cell junction are the most susceptible to HPV-induced cell transformation (Jastreboff and Cymet, 2002; Jordan and Monaghan, 2004; Ponten and Guo, 1998). An analogous cell type apparently does not exist in men. However, despite lack of studies on the subject, it would be assumed that men are transmitters of HPVs (Baldwin *et al.*, 2003; Dunne *et al.*, 2006; Giuliano *et al.*, 1999). Predictably, the incidence of penile cancers increases dramatically in individuals who are HIV positive (Aboulafia and Gibbons, 2001; Arany and Tyring, 1998; Laurence, 2003; Palefsky and Barrasso, 1996; Sirera *et al.*, 2006; von Krogh *et al.*, 1995), which likely reflects the overall increase incidence of HPV infections detected in women.

Anal cancer is a relatively rare disease, ~80% of which are HPV positive (Gloeckler Ries *et al.*, 2003; Hankey *et al.*, 1999; Zippin and Lum, 1993). Anal cancer amounts to about 4% of all digestive tract cancers. The incidence of anal cancers in women is slightly higher than in men (Gloeckler Ries *et al.*, 2003; Hankey *et al.*, 1999; Zippin and Lum, 1993), which could be indicative of the overall higher rate of HPV infection among women. It appears that the incidence of anal cancers are rising in the past 10 years, reflecting changes in sexual behavior (http://seer.cancer.gov/) (Gloeckler Ries *et al.*, 2003). The highest level of risk for anal cancer caused by HPV is associated with MSM (Piketty *et al.*, 2004). The rate of anal cancer among HIV-negative heterosexual men is ~1.3/100,000, while the rate of anal cancers in HIV-negative MSM is 35/100,000. Among MSM who are HIV positive, the rate of anal cancer is twofold higher than HIV-negative MSM (Fakhry and Gillison, 2006).

HPV infections of the conjunctiva of the eye are more common than previously appreciated, although resulting HPV-induced tumors of

the conjunctiva are very rare (Mincione *et al*., 1992; Reszec and Sulkowski, 2005; Tabrizi *et al*., 1997; Waddell *et al*., 2003). Though the incidence of eye or eye-orbit tumors in the United States is extremely low (<1/100,000) (Gloeckler Ries *et al*., 2003), in African countries, conjunctival tumors are more common and are undoubtedly influenced by nutrition, additional disease burdens, but most obviously by the relatively high impact of HIV in Africa (Ateenyi-Agaba *et al*., 2006; Frisch *et al*., 2000; Goedert, 2000; Newton *et al*., 2002; Waddell *et al*., 1996, 2003).

Given the wide range of cancers caused by HPV, the recent development of an HPV vaccine provides some hope for providing protection against many of the cancers described earlier. However, vaccine efforts have concentrated on only two high-risk strains (HPV16 and HPV18) (Mao *et al*., 2006; Villa *et al*., 2005), while there are at least 15 known high-risk strains. We also have evidence that multiple regional HPV variants exist, particularly in Africa, for which, the extent of protection by the current vaccine is unknown (Calleja-Macias *et al*., 2004; Chan *et al*., 1992; Ong *et al*., 1993; Touze *et al*., 1998; Williamson *et al*., 1994; Xi *et al*., 1998). Furthermore, the distribution of high-risk HPVs varies from country to country (Calleja-Macias *et al*., 2004; Chan *et al*., 1992; De Vuyst *et al*., 2003; Munoz *et al*., 2004; Ong *et al*., 1993; Williamson *et al*., 1994; Xi *et al*., 1998). Therefore, it is important to take a long-range view of prevention of HPV-induced cancers by use of vaccination strategies that take into account variants.

VII. HPV—The Causative Agent

A. Papillomavirus Genome Structure

HPVs are a family of small, nonenveloped, double-strand DNA viruses that establish a persistent infection, which may remain subclinical in the skin or genital tract for up to 10–20 years, but can often cause acute warts. Papillomavirus genomes are small circular DNA of 8 kb, which encodes eight major proteins. As is typical for DNA viruses, the immediate early genes (E6 and E7) are involved with taking over the cell cycle (Fig. 1) (Howley, 1996; Howley *et al*., 1989; zur Hausen, 1999, 2000). Unlike more complex viruses like herpesviruses, HPVs use the strategy of replicating at low copy, and thus, do not carry their own polymerase gene. Instead, gene products encoded by E1 and E2 recruit cellular polymerase α to the viral origin (Frattini and Laimins, 1994; Howley, 1996; Howley *et al*., 1989; Sedman and Stenlund, 1995). The genome is simply organized into early and late genes, with only two capsid genes, L1 and L2.

The functions of the viral proteins are well established and are summarized below (Table II).

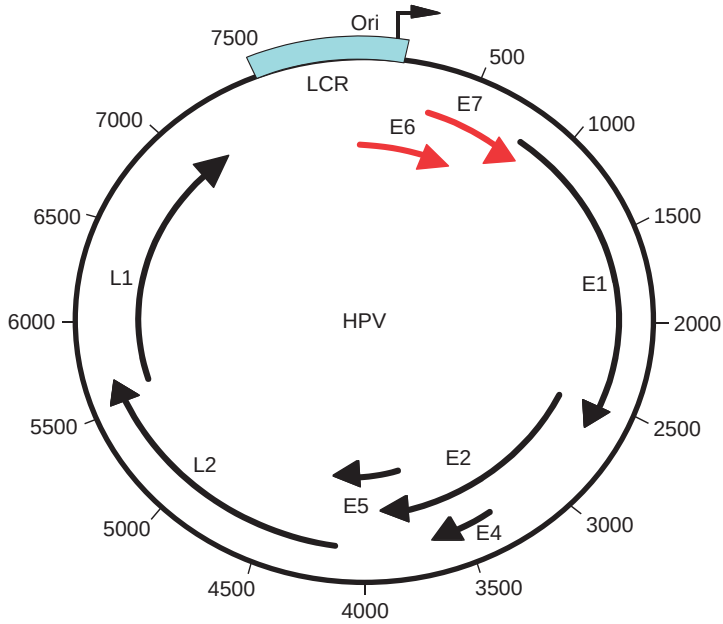


FIGURE 1 The HPV genome is a 7.9 kb double-stranded circular genome. The genome is controlled by a single keratin-dependent promoter element; the long control region (LCR; in blue). At the 3′ end of the LCR is the origin of replication (*nucleotide position 1*). E6, E7 (red), and E5 are viral oncogenes; E1 and E2 early genes encode replication proteins. The E4 ORF is actually expressed early and late in the viral life cycle. The late genes, L1 and L2, are the major and minor capsid genes, respectively. The viral protein functions are detailed in Table I.

B. The HPV Life Cycle

HPVs initiate their life cycle by gaining access to basal keratinocytes of the stratified epithelium; either skin or mucosa through a site of wounding (Fig. 2). Papillomavirus DNA replication is closely coupled to the process of keratinocyte differentiation in infected squamous epithelium (reviewed in Chow and Broker, 1994). In the basal and parabasal epithelial cells, HPV is maintained as a low-copy number episome (5–50 copies per cell) that undergoes regulated DNA replication under the control of viral and host proteins. As infected keratinocytes differentiate and enter the stratum spinosum layer of the epithelium, there is a coincident increase in concentration of E1 and E2 proteins (for review, see Shaw and Howley, 2001). Induction of vegetative replication is consistent with a mode switch from theta to rolling-circle replication mechanisms (Flores and Lambert, 1997). As a consequence of this rolling-circle DNA replication mechanism, multiple rounds of viral DNA synthesis occurs in a given S-phase of the host keratinocyte (Hoffmann *et al.*, 2006), and an increase in copy number up to between 100 and 1000 copies

TABLE II The Gene Products Encoded in the HPV Genome

Designation	*Function*
E6	Viral oncoprotein; functions by binding and degrading p53 tumor suppressor via activity of E6AP, ubiquitin ligase, resulting ubiquitin pathway-mediated degradation of p53, preventing apoptosis
E7	Nuclear oncoprotein; functions by binding and degrading of pRb family tumor suppressor products. This causes release of E2F that induces expression of S-phase-related genes
E1	An ATP-dependent DNA helicase; has double and single strand DNA-binding activities. Required for viral DNA replication; interacts with E2 protein and with DNA polymerase α. Significant structural homology to SV40 large T antigen (Clertant and Seif, 1984; Mansky *et al*., 1997; Seif, 1984). Sequence-specific DNA binding by E1 is mediated by the papillomavirus E2 protein; association of E1 with E2 enhances the affinity of E1 for DNA (Frattini and Laimins, 1994; Sedman and Stenlund, 1995)
E2	Binds a 12 base pair palindromic sequence: $ACCG(A)_4CGGT$ in the Ori and functions as a transcriptional activator; binds and recruits the E1 protein that stimulates initiation of DNA replication; E2 also functions in HPV genome maintenance by tethering newly synthesized DNAs to chromosomes during mitosis, which allows equal partitioning to daughter cells
E4	Expressed as a late protein; blocks cell cycle at G2/M; induces collapse of cytokeratin network; possibly promoting virus release; poorly conserved across HPV types
E5	A viral oncoprotein; binds EGF receptor; able to transform rodent cells in culture; membrane associated; functions include binding to β receptor for platelet-derived growth factor
L1	Major capsid protein
L2	Minor capsid protein

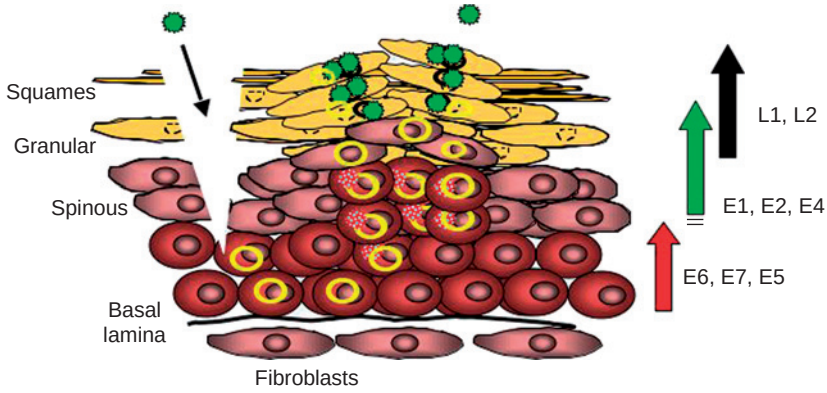


FIGURE 2 The HPV life cycle. Virions enter the stratified epithelium through a site of wounding, where they gain access to the mitotically active basal-layer keratinocytes. During the maintenance phase, expression of E6, E7, and E5 induces cell proliferation, and the viral genome is replicated extrachromosomally at low-copy number (5–50 copies per cell). As the cells differentiate, the expression level of E1, E2, and E4 increases in the spinous layer. A transition from theta to rolling-circle replication results in an increase in copy number up to 100–1000 copies per cell. Postamplification, high levels of L1 and L2 capsid genes are expressed and capsid assembly occurs in the granular and squamous layers of the stratified epithelium. Progeny virus is released by desquamation.

per cell. Vegetative HPV DNA replication requires the virus-encoded E1 (a DNA helicase per ATPase) and E2 (a transcriptional *trans*-modulator) proteins, and initiates at the E1 binding site palindrome near the 5′ end of the viral long control region (Kuo *et al.*, 1994).

C. The HPV Capsid and the Vaccine

Capsid assembly of HPVs occurs in the more terminally differentiated layers of the stratified epithelium (Fig. 2). HPVs have icosahedral capsids arranged in a $T = 7d$ lattice (Baker *et al.*, 1991). The capsids are made up of 360 L1 molecules organized into 72 pentamers. Disulfide bond interactions between L1 molecules are important in particle assembly and disassembly (Li *et al.*, 1998). There are 12 L2 minor capsid proteins that are associated with the inner surface of the L1 pentamers (Belnap *et al.*, 1996). High resolution cryoelectron microscopic structures of bovine papillomavirus (BPV) have been achieved (Baker *et al.*, 1991); less is known about HPV structure, assembly, and uncoating. L1 expression is sufficient to allow self-assembly of virus-like particles (VLPs) in the absence of other viral components (Casini *et al.*, 2004). However, L2, when coexpressed with L1, intercalates into L1 VLPs and appears to be required for virion infectivity (Kawana *et al.*, 2001; Stauffer *et al.*, 1998). Though the precise role of L2 during infection is not clear, it may nucleate L1-pentamer formation.

The simple, nonenveloped icosahedral HPV virions lend themselves to vaccine development. The recently developed quadrivalent vaccine targets two high-risk HPVs (16 and 18) and two low-risk HPVs (6 and 11). However, there is little evidence of significant cross-protection against the other 14 oncogenic HPVs. Furthermore, there are at least 40 genital HPVs. Thus, the development of vaccines that have a wider cross-protection or tailoring HPV vaccines for different regions of the world will become necessary.

D. Epidemiology of HPV and HIV/AIDS

Epidemiological evidence gathered over several years has determined that 15–20 of the 40 of the mucosal HPV types are associated with a higher risk of progression to cervical cancer (24 and 29). The frequency of individual high-risk HPV types worldwide has been shown to vary in respect to major global regions such as Asia, Europe, North America, South America, and sub-Saharan Africa (5, 14, and 23). The rate of genital HPVs in the United States, as detected by PCR of the L1 region, is ~39.2% (Peyton *et al.*, 2001). Estimates of the rates of HPV in Africa vary from 14% to 60% depending on the country, the coincident STDs, and the methods of detection (Czegledy *et al.*, 1992; Gravitt *et al.*, 2002; Hassen *et al.*, 2003; Langley *et al.*, 1996; Mayaud *et al.*, 2001; Motti *et al.*, 1996; Nzila *et al.*, 1991; O'Farrell *et al.*, 1989; Ong *et al.*, 1993; Serwadda *et al.*, 1999; St. Louis *et al.*, 1993; Thomas *et al.*, 2004; Waddell *et al.*, 1996; Williamson *et al.*, 2002). The rate of HPV infections in HIV-positive patients in Zambia are very high, though rather little data is available (Mosunjac *et al.*, 2003; Patil *et al.*, 1995). As a whole, sub-Saharan Africa has the among the highest rates of cervical cancer in the world (Bailie *et al.*, 1996; Clarke and Chetty, 2002; Langley *et al.*, 1996; ter Meulen *et al.*, 1992; Williamson *et al.*, 2002). The distribution of oncogenic HPVs in Africa differs from the United States and Europe. For example, studies done by Nubia Munoz have pointed out that in Nigeria, the most prevalent oncogenic HPV is HPV35, not HPV16, which is the most common in the United States and Europe (Thomas *et al.*, 2004). Several studies have described HPV variants unique to Africa (Calleja-Macias *et al.*, 2004; Chan *et al.*, 1992; Ong *et al.*, 1993; Williamson *et al.*, 1994). This points to a need to further investigate HPV variants in terms of HPV pathogenesis and a potential need to expand the selection of vaccine targets.

The rates of HIV infection in urban area along a contiguous stretch from Uganda to Botswana and South Africa have continued to climb in recent years (Morison, 2001). Several studies have addressed HIV-related malignancies associated with HHV-8 (KSHV) and EBV, which are associated with increased morbidity (Ablashi *et al.*, 1999; Contreras *et al.*, 1997; Lazzi *et al.*, 1998; Parkin *et al.*, 2000; Sapp *et al.*, 2001). Despite frequency variation, HPV16 infection has been shown to be more prevalent than any other high-risk HPV type in most regions of the world. However,

HIV-positive populations have a much higher rate of HPV16 positive tumors than most HIV-negative populations (1, 2, 9, 18, and 28). Thus, the incidence of high-risk HPV-malignancies is amplified by HIV immune suppression.

It is clear that impaired cell-mediated and humoral immunity influences the advancement of high-risk HPVs in HIV-positive individuals. Since the pool of memory and effector T cells can be dramatically shifted in HIV-positive individuals (even those who do not have AIDS), it would be expected that a certain degree of derepression of HPV replication would occur as well as a lack of adequate surveillance preneoplastic lesions. Several studies have shown a strong and consistent association between HIV and HPV coinfection and the development of cervical intraepithelial neoplasia (CIN) and genital cancer (7, 11, 13, and 15). There is evidence to show that HIV-positive women have a significantly higher rate of CIN than their counterparts and are more likely to progress to invasive carcinoma than HIV-negative women (4, 12, and 20). A recent study in Brazil has shown that a very high proportion of HIV-infected women is infected with HPV and often carries multiple HPV genotypes (15).

A relationship between the HIV and HPV pathogenesis has been investigated by several studies (Durante *et al.*, 2003; Heard *et al.*, 2004; Klencke and Palefsky, 2003; Massad *et al.*, 2004; Palefsky, 1991, 2003; Piketty *et al.*, 2003, 2004; Silverberg *et al.*, 2002; Strickler *et al.*, 2003; Williams *et al.*, 1994). For example, a study by Silverberg *et al.* (2002) found that HIV-seropositive women were 3.2-fold more likely to present with genital warts than HIV-seronegative women. Malignancies as complications are an increasing cause of morbidity of HIV-infected individuals (Patil *et al.*, 1995; Thomas, 2001). The EBV-induced malignancy, NHL, occurs at a rate of 2.9% in AIDS patients, ~60 times the average in non-AIDS patients (Beral *et al.*, 1991). Occurrences of AIDS-related KS still occur at elevated levels, but recent advances in detection and stage analysis has improved the prognosis for HIV-positive patients (Quinlivan *et al.*, 2002). Likewise, an improved understanding of the HPV disease process, as it relates to HIV, is essential, since antiretroviral therapy in HIV-positive individuals has not been shown to effectively reverse HPV-related disease.

The association of malignancies, such as NHL and KS, has been recognized since the beginning of the HIV epidemic, and KS is the neoplasm most commonly found in people infected with HIV. These neoplasms are responsible for extensive morbidity and mortality. In Zambia and other sub-Saharan nations, cervical cancer is the most common cancer (Baay *et al.*, 2004; Hawes *et al.*, 2003; Xi *et al.*, 1998, 2003). Although in Africa, public education campaigns about STDs and condoms have been instituted in urban areas, there has been little success in poorer rural areas (Agha and Kusanthan, 2003). Thus, combined with endemic HIV, a high prevalence of high-risk HPVs presents a great risk for progression of dysplasias to cancer.

Infection by high-risk HPVs, especially HPV16, can induce warts; low-grade dysplasias, CIN, CIN 1 and CIN 2 designations are reversible forms of precancerous lesions (Fig. 3). Integration of the HPV genome can lead to an increase of expression of E6 and E7 resulting in progression to CIN 3, which is irreversible. Accumulation of mutations in cellular genes results in permanent changes in cell character leading to invasive carcinoma *in situ*. Progression from a benign cervical lesion to invasive cervical cancer usually occurs years after infection.

Cervical cancer and precancerous lesions (CIN) are now the most common cancer-related affliction affecting women in sub-Saharan Africa and other developing countries in the world. The rates of cervical cancer in Africa are fourfold higher in than in North America and Europe.

E. The Mechanism of HPV-Induced Transformation and Cancer Progression

High-risk HPVs (types 16, 18, and 31 e.g.) are often found integrated into the host genome (Yee *et al*., 1985a). The integrated state of the viral genome is not supportive of the viral life cycle, but can confer a growth advantage to cells due to increased expression of E6 and E7 (Jeon *et al*.,

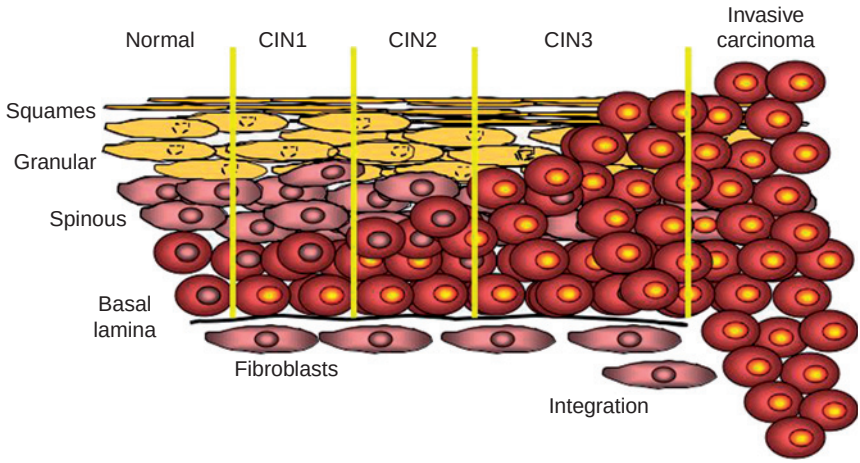


FIGURE 3 Progression from a benign cervical lesion to invasive cervical cancer. In the diagram, HPV-positive cells are depicted by yellow nuclei. Infection by oncogenic HPV types, especially HPV16, can cause formation of a benign wart, low or high-grade dysplasia. CIN 1 and CIN 2 designations are reversible forms of precancerous lesions and CIN 3 is irreversible. Carcinoma *in situ* occurs many years after an infection. This results from the effects of HPV genes, particularly those encoding E6 and E7, which are the two viral oncoproteins that are preferentially retained and expressed in cervical cancers by integration of the viral DNA into the host genome.

1995). The common feature in cancers is the expression of E6 and E7 genes which functionally inactivate p53 and Rb, respectively (Durst *et al.*, 1987; Howley *et al.*, 1989; Munger *et al.*, 1989) (Fig. 4). In oncogenic HPV strains, E6 and E7 oncoproteins can block the negative growth signaling pathways of the cell via interactions with p53 and pRB tumor suppressor proteins. As a result, high-risk HPV-infected cells proliferation become disregulated and then, transformation develops. The full-length HPV E6 genes encode a 160-amino acids protein, which contains two domains including zinc binding Cys-X-X-Cys motifs. High-risk HPV E6 proteins both have antiapoptotic activities and can interfere with the antiproliferative functions of p53, the cellular tumor suppressor. For this to occur, E6 first forms a complex with a cellular ubiquitin-protein ligase E6AP, the E6/E6AP complex then acts as a p53-specific ubiquitin-protein ligase to accelerate degradation of p53. E6 is also known to induce expression of human telomerase (htert), leading to the functional outcome of increased life span of infected keratinocytes. Upregulation of htert is also hallmark of a number of human cancers. Although the mechanism by which E6 suppresses cell death is established, relatively little is known about the localization of E6 proteins and its related splice products and how this relates to these functional interactions. Furthermore, the

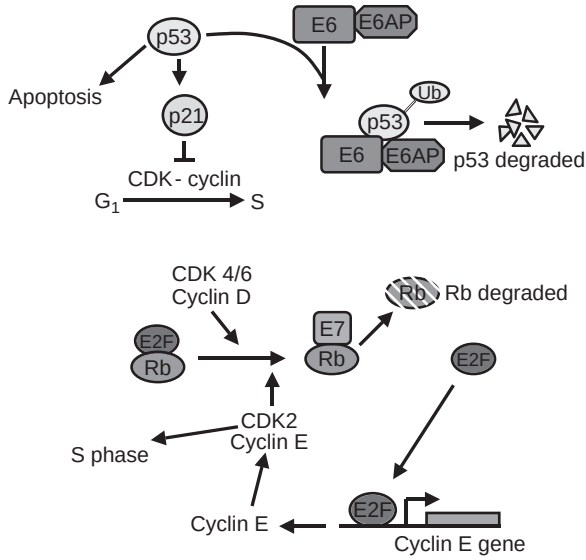


FIGURE 4 Diagram of the role of E6 and E7 in disregulation of the cell cycle. Expression of E6 leads to recruitment of E6AP (a ubiquitin ligase). This complex causes degradation of p53, which then inhibits the p21-dependent block of the G_1 to S transition. Similarly, E7 binding to Rb displaces E2F, resulting in Rb's degradation. E2F can then activate expression of cyclin E and other S-phase related gene products.

localization of E6AP in normal and E6-expressing cells is essentially unknown. Also, unknown is whether interactions and localization changes between E6 and htert alter E6 function.

E7 binds Rb family member proteins resulting in their displacement from E2F and eventual degradation. Release of E2F allows it to freely activate S-phase related genes responsible for the G1 to S transition. Just as E6 causes inactivation of p53, allowing unchecked DNA synthesis, E7, by releasing E2F, activates the expression of genes required for cellular DNA synthesis. Even if the cellular DNA is damaged, the lack of p53 allows the cell to survive through an E7-induced S-phase and replicate the viral genome.

The rate of advancement of HPV lesions, from benign hyperplasia to carcinoma *in situ*, is affected by additional factors, which includes immunocompetence. HIV status, directly affects immune status which determines susceptibility to secondary infections, including HPV. In addition, progression of HPV tumors are affected by HIV status since surveillance of cancer cells is impaired. It is well established that cofactors in addition to immune status, such as alcohol, drugs, smoking, oral contraceptives, and hormone levels influence HPV infection and progression of HPV-induced cancers (Fig. 5). The ability of E6 and E7 of high-risk HPVs to inactivate p53 and pRb directly correlates with the probability to develop tumors. HPV coin-

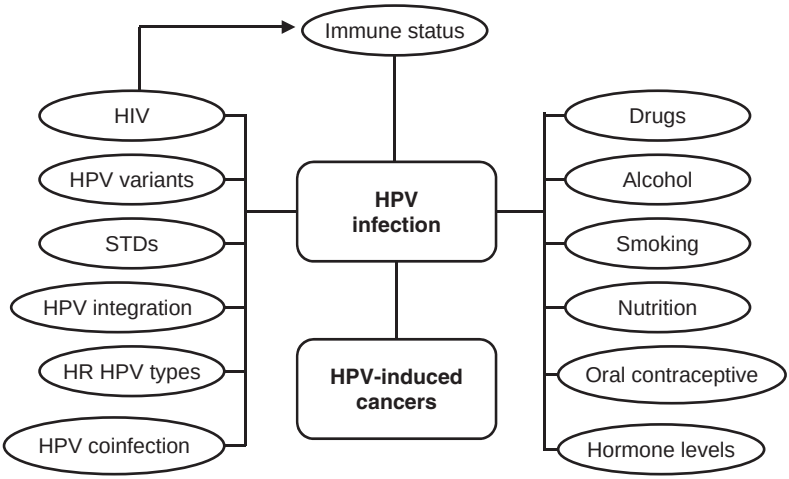


FIGURE 5 The rate of advancement of HPV lesions, from benign hyperplasia to carcinoma *in situ*, is affected by additional factors, which includes immunocompetence. HIV status, alcohol, drugs, smoking, oral contraceptives, and hormone levels influence HPV infection and progression of HPV-induced cancers. High-risk HPVs, HPV coinfection, variants, genome integration, and infection of other STDs affect the propensity for HPV-induced cancer to occur and progress.

fection, variants, genome integration, as well as other STDs affect the propensity for HPV-induced cancer to occur and progress.

HPV-related diseases are common causes of morbidity and mortality, both in the United States and worldwide. During the year of 2006, the American Cancer Society (ACS) estimated that there were 9710 new cases of cervical carcinoma and 3700 cervical cancer deaths in the United States. The ACS estimates that there were 4660 new anal cancers with 660 deaths and 1470 new penile cancers with 280 deaths (ACS, 2006). There were ~6000 new cases of oral and pharyngeal cancers with 1400 deaths are attributable to HPV infection. In developing countries, the second greatest cancer cause of death among women is cervical cancer. Still, there are few effective antiviral therapies for prevention or treatment of HPV-related diseases. Furthermore, while the quadrivalent VLP HPV vaccine has the long-term potential to reduce HPV-induced cancers by 70%, population-based studies indicate that, until all girls are immunized prior to the onset of sexual activity, the vaccine will prevent only 30–50% of cervical malignancies. More of a concern is the lack of availability of the prophylactic vaccine in countries **which** are afflicted with high rates of cervical cancer. Thus, we will continue to face a great deal of cervical cancer morbidity and mortality in the years to come. We are far from eliminating the need for treatments for HPV infection and HPV-induced anogenital dysplasias and cancers. Hence, the continuing need for research into papillomavirus pathogenesis especially in the context of the ongoing HIV crisis.

VIII. Conclusions

AIDS malignancies have been a major complication of the HIV disease course, and this is likely to continue in HIV-infected individuals. In the era of HAART therapy, the survival rate of the HIV-infected individuals has increased dramatically mainly because of the suppression of HIV viral load and the restoration of the immune response. However, even though HAART appears to be effective, still only leads to partial immune reconstitution. Prolonged immunosuppression will likely lead to a resurgence of AIDS-associated cancers. This coupled with the fact that there are still over 40 million individuals living with HIV today, many of whom are located in regions of the world where HAART is still not widely available, such as the African continent. It is expected that AIDS-associated cancers will continue to pose a major challenge globally for many years to come. As described earlier in this chapter, many of the cancers associated with immunosuppressed individuals are those that were found to have viral etiology. Other than the development and refinement of effective vaccines against these viruses, as in the case of HPV, there is a need for a better understanding on the role of oncogenic viral cofactor in the disease, the potential mechan-

isms, the viral genes and the host immune response that are involved. This knowledge will lead to the development of better strategies that could prevent infection and the malignant transformation by these potentially oncogenic viruses.

Acknowledgments

This publication was made possible, in part, by support from the following NIH grants: PHS award CA76958, NCRR COBRE grant RR15635, and INBRE grant P20 RR016469. The authors also wish to acknowledge Ms. Dianna Wright for help with the preparation of this chapter.

References

Aaltonen, L. M., Cajanus, S., Back, L., Nieminen, P., Paavonen, J., and Ranki, A. (2005). Extralaryngeal HPV infections in male patients with adult-onset laryngeal papillomatosis. *Eur. Arch. Otorhinolaryngol.* **262**(9), 708–712.

Ablashi, D., Chatlynne, L., Cooper, H., Thomas, D., Yadav, M., Norhanom, A. W., Chandana, A. K., Churdboonchart, V., Kulpradist, S. A., Patnaik, M., Liegmann, K., Masood, R., *et al.* (1999). Seroprevalence of human herpesvirus-8 (HHV-8) in countries of Southeast Asia compared to the USA, the Caribbean and Africa. *Br. J. Cancer* **81**(5), 893–897.

Aboulafia, D. M., and Gibbons, R. (2001). Penile cancer and human papilloma virus (HPV) in a human immunodeficiency virus (HIV)-infected patient. *Cancer Invest.* **19**(3), 266–272.

ACS (2006). Cancer facts and figures. American Cancer Society: www.cancer.org.

Agha, S., and Kusanthan, T. (2003). Equity in access to condoms in urban Zambia. *Health Policy Plan.* **18**(3), 299–305.

Altmann, M., Pich, D., Ruiss, R., Wang, J., Sugden, B., and Hammerschmidt, W. (2006). Transcriptional activation by EBV nuclear antigen 1 is essential for the expression of EBV's transforming genes. *Proc. Natl. Acad. Sci. USA* **103**(38), 14188–14193.

Arany, I., and Tyring, S. K. (1998). Systemic immunosuppression by HIV infection influences HPV transcription and thus local immune responses in condyloma acuminatum. *Int. J. STD AIDS* **9**(5), 268–271.

Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C., and Cesarman, E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature* **385**(6614), 347–350.

Ateenyi-Agaba, C., Weiderpass, E., Tommasino, M., Smet, A., Arslan, A., Dai, M., Katongole-Mbidde, E., Hainaut, P., Snijders, P. J., and Franceschi, S. (2006). Papillomavirus infection in the conjunctiva of individuals with and without AIDS: An autopsy series from Uganda. *Cancer Lett.* **239**(1), 98–102.

Baay, M. F., Kjetland, E. F., Ndhlovu, P. D., Deschoolmeester, V., Mduluza, T., Gomo, E., Friis, H., Midzi, N., Gwanzura, L., Mason, P. R., Vermorken, J. B., and Gundersen, S. G. (2004). Human papillomavirus in a rural community in Zimbabwe: The impact of HIV co-infection on HPV genotype distribution. *J. Med. Virol.* **73**(3), 481–485.

Bailey, P. (1929). Intracranial sarcomatous tumors of leptomeningeal origin. *Arch. Surg.* **18**, 1359–1402.

Bailie, R. S., Selvey, C. E., Bourne, D., and Bradshaw, D. (1996). Trends in cervical cancer mortality in South Africa. *Int. J. Epidemiol.* **25**(3), 488–493.

Bais, C., Van Geelen, A., Eroles, P., Mutlu, A., Chiozzini, C., Dias, S., Silverstein, R. L., Rafii, S., and Mesri, E. A. (2003). Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/KDR. *Cancer Cell* **3**(2), 131–143.

Baker, T. S., Newcomb, W. W., Olson, N. H., Cowsert, L. M., Olson, C., and Brown, J. C. (1991). Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys. J.* **60**(6), 1445–1456.

Baldwin, S. B., Wallace, D. R., Papenfuss, M. R., Abrahamsen, M., Vaught, L. C., Kornegay, J. R., Hallum, J. A., Redmond, S. A., and Giuliano, A. R. (2003). Human papillomavirus infection in men attending a sexually transmitted disease clinic. *J. Infect. Dis.* **187**(7), 1064–1070.

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* **284**(5414), 641–644.

Bayley, A. C. (1991). Occurrence, clinical behaviour and management of Kaposi's sarcoma in Zambia. *Cancer Surv.* **10**, 53–71.

Belnap, D. M., Olson, N. H., Cladel, N. M., Newcomb, W. W., Brown, J. C., Kreider, J. W., Christensen, N. D., and Baker, T. S. (1996). Conserved features in papillomavirus and polyomavirus capsids. *J. Mol. Biol.* **259**(2), 249–263.

Beral, V. (1991). Epidemiology of Kaposi's sarcoma. *Cancer Surv.* **10**, 5–22.

Beral, V., Peterman, T., Berkelman, R., and Jaffe, H. (1991). AIDS-associated non-Hodgkin lymphoma. *Lancet* **337**(8745), 805–809.

Besson, C., Goubar, A., Gabarre, J., Rozenbaum, W., Pialoux, G., Chatelet, F. P., Katlama, C., Charlotte, F., Dupont, B., Brousse, N., Huerre, M., Mikol, J., *et al.* (2001). Changes in AIDS-related lymphoma since the era of highly active antiretroviral therapy. *Blood* **98**(8), 2339–2344.

Biggar, R. J., and Rabkin, C. S. (1996). The epidemiology of AIDS—related neoplasms. *Hematol. Oncol. Clin. North Am.* **10**(5), 997–1010.

Bosch, F. X., Durst, M., Schwarz, E., Boukamp, P., Fusenig, N. E., and zur Hausen, H. (1991). The early genes E6 and E7 of cancer associated human papilloma viruses as targets of tumor suppression? *Behring Inst. Mitt.* **89**, 108–121.

Boshoff, C., Endo, Y., Collins, P. D., Takeuchi, Y., Reeves, J. D., Schweickart, V. L., Siani, M. A., Sasaki, T., Williams, T. J., Gray, P. W., Moore, P. S., Chang, Y., and Weiss, R. A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science* **278**(5336), 290–294.

Brennan, P., Floettmann, J. E., Mehl, A., Jones, M., and Rowe, M. (2001). Mechanism of action of a novel latent membrane protein-1 dominant negative. *J. Biol. Chem.* **2001**, 1195–1203.

Brennan, P., Mehl, A. M., Jones, M., and Rowe, M. (2002). Phosphatidylinositol 3-kinase is essential for the proliferation of lymphoblastoid cells. *Oncogene* **21**(8), 1263–1271.

Brockmeyer, N., and Barthel, B. (1998). Clinical manifestations and therapies of AIDS associated tumors. *Eur. J. Med. Res.* **3**(3), 127–147.

Cahir-McFarland, E. D., Davidson, D. M., Schauer, S. L., Duong, J., and Kieff, E. (2000). NF-kappaB inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA* **97**, 6055–6060.

Callahan, J., Pai, S., Cotter, M., and Robertson, E. S. (1999). Distinct patterns of viral antigen expression in Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus coinfected body-cavity-based lymphoma cell lines: Potential switches in latent gene expression due to coinfection. *Virology* **262**(1), 18–30.

Calleja-Macias, I. E., Kalantari, M., Huh, J., Ortiz-Lopez, R., Rojas-Martinez, A., Gonzalez-Guerrero, J. F., Williamson, A. L., Hagmar, B., Wiley, D. J., Villarreal, L., Bernard, H. U., *et al.* (2004). Genomic diversity of human papillomavirus-16, 18, 31, and 35 isolates in a Mexican population and relationship to European, African, and Native American variants. *Virology* **319**(2), 315–323.

Carbone, A., and Gloghini, A. (2005). AIDS-related lymphomas: From pathogenesis to pathology. *Br. J. Haematol.* **130**(5), 662–670.

Casini, G. L., Graham, D., Heine, D., Garcea, R. L., and Wu, D. T. (2004). *In vitro* papillomavirus capsid assembly analyzed by light scattering. *Virology* **325**(2), 320–327.

Cates, W., Jr., and Dallabetta, G. (1999). The staying power of sexually transmitted diseases. *Lancet* **354**(Suppl. SIV62).

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* **332**(18), 1186–1191.

Cesarman, E., Nador, R. G., Bai, F., Bohenzky, R. A., Russo, J. J., Moore, P. S., Chang, Y., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. *J. Virol.* **70**(11), 8218–8223.

Challine, D., Roudot-Thoraval, F., Sarah, T., Laperche, L., Boisson, B., Mauberquez, S., Dubernet, F., Rigot, P., Lefrere, F., Mercier, B., Brossard, Y., Rouet, F., *et al.* (2001). Seroprevalence of human herpes virus 8 antibody in populations at high or low risk of transfusion, graft, or sexual transmission of viruses. *Transfusion* **41**(9), 1120–1125.

Chan, S. Y., Ho, L., Ong, C. K., Chow, V., Drescher, B., Durst, M., ter Meulen, J., Villa, L., Luande, J., and Mgaya, H. N. (1992). Molecular variants of human papillomavirus type 16 from four continents suggest ancient pandemic spread of the virus and its coevolution with humankind. *J. Virol.* **66**(4), 2057–2066.

Chang, Y., and Moore, P. S. (1996). Kaposi's sarcoma (KS)-associated herpesvirus and its role in KS. *Infect. Agents Dis.* **5**(4), 215–222.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**(5192), 1865–1869.

Chang, H., Gwack, Y., Kingston, D., Souvlis, J., Liang, X., Means, R. E., Cesarman, E., Hutt-Fletcher, L., and Jung, J. U. (2005). Activation of CD21 and CD23 gene expression by Kaposi's sarcoma-associated herpesvirus RTA. *J. Virol.* **79**(8), 4651–4663.

Cheah, P. L., and Looi, L. M. (1998). Biology and pathological associations of the human papillomaviruses: A review. *Malays. J. Pathol.* **20**(1), 1–10.

Chen, H., Hutt-Fletcher, L., Cao, L., and Hayward, S. D. (2003). A positive autoregulatory loop of LMP1 expression and STAT activation in epithelial cells latently infected with Epstein-Barr virus. *J. Virol.* **77**(7), 4139–4148.

Cheung, T. W. (2004). AIDS-related cancer in the era of highly active antiretroviral therapy (HAART), a model of the interplay of the immune system, virus, and cancer. "On the offensive—the Trojan Horse is being destroyed"—Part B: Malignant lymphoma. *Cancer Invest.* **22**(5), 787–798.

Chow, L. T., and Broker, T. R. (1994). Papillomavirus DNA replication. *Intervirology* **37**(3–4), 150–158.

Cingolani, A., Fratino, L., Scoppettuolo, G., and Antinori, A. (2005). Changing pattern of primary cerebral lymphoma in the highly active antiretroviral therapy era. *J. Neurovirol.* **11**(Suppl. 3), 38–44.

Clarke, B., and Chetty, R. (2002). Postmodern cancer: The role of human immunodeficiency virus in uterine cervical cancer. *Mol. Pathol.* **55**(1), 19–24.

Clarke, J., Terry, R. M., and Lacey, C. J. (1991). A study to estimate the prevalence of upper respiratory tract papillomatosis in patients with genital warts. *Int. J. STD AIDS* **2**(2), 114–115.

Clertant, P., and Seif, I. (1984). A common function for polyoma virus large-T and papillomavirus E1 proteins? *Nature* **311**(5983), 276–279.

Clifford, G. M., Polesel, J., Rickenbach, M., Dal Maso, L., Keiser, O., Kofler, A., Rapiti, E., Levi, F., Jundt, G., Fisch, T., Bordoni, A., De Weck, D., *et al.* (2005). Cancer risk in the Swiss HIV Cohort Study: Associations with immunodeficiency, smoking, and highly active antiretroviral therapy. *J. Natl. Cancer Inst.* **97**(6), 425–432.

Cole, M. D., and McMahon, S. B. (1999). The Myc oncoprotein: A critical evaluation of transactivation and target gene regulation. *Oncogene* **18**(19), 2916–2924.

Contreras, A., Falkler, W. A., Jr., Enwonwu, C. O., Idigbe, E. O., Savage, K. O., Afolabi, M. B., Onwujekwe, D., Rams, T. E., and Slots, J. (1997). Human herpesviridae in acute necrotizing ulcerative gingivitis in children in Nigeria. *Oral Microbiol. Immunol.* **12**(5), 259–265.

Cook, J. (1962). The clinical features of Kaposi's sarcoma in the East African Bantu. *Acta Unio Int. Contra Cancrum* **18**, 388–393.

Cotter, M. A., 2nd, Subramanian, C., and Robertson, E. S. (2001). The Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen binds to specific sequences at the left end of the viral genome through its carboxy-terminus. *Virology* **291**(2), 241–259.

Cunha, A. M., Caterino-de-Araujo, A., Costa, S. C., Santos-Fortuna, E., Boa-Sorte, N. C., Goncalves, M. S., Costa, F. F., and Galvao-Castro, B. (2005). Increasing seroprevalence of human herpesvirus 8 (HHV-8) with age confirms HHV-8 endemicity in Amazon Amerindians from Brazil. *J. Gen. Virol.* **86**(Pt 9), 2433–2437.

Czegledy, J., Rogo, K. O., Evander, M., and Wadell, G. (1992). High-risk human papillomavirus types in cytologically normal cervical scrapes from Kenya. *Med. Microbiol. Immunol. (Berl.)* **180**(6), 321–326.

Dawson, C. W., Rickinson, A. B., and Young, L. S. (1990). Epstein-Barr virus latent membrane protein inhibits human epithelial cell differentiation. *Nature* **344**(6268), 777–780.

de Sanjose, S., Marshall, V., Sola, J., Palacio, V., Almirall, R., Goedert, J. J., Bosch, F. X., and Whitby, D. (2002). Prevalence of Kaposi's sarcoma-associated herpesvirus infection in sex workers and women from the general population in Spain. *Int. J. Cancer* **98**(1), 155–158.

Devergne, O., Hatzivassiliou, E., Izumi, K., Kaye, K., Kleijnen, M., Kieff, E., and Mosialos, G. (1996). Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B lymphocyte transformation: Role in NF-κB activation. *Mol. Cell Biol.* **16**, 7098–7108.

Devergne, O., McFarland, E. C., Mosialos, G., Izumi, K. M., Ware, C. F., and Kieff, E. (1998). Role of the TRAF binding site and NF-kappaB activation in Epstein-Barr virus latent membrane protein 1-induced cell gene expression. *J. Virol.* **72**, 7900–7908.

de Villiers, E. M., Weidauer, H., Le, J. Y., Neumann, C., and zur Hausen, H. (1986). (Papilloma viruses in benign and malignant tumors of the mouth and upper respiratory tract). *Laryngol. Rhinol. Otol. (Stuttg)* **65**(4), 177–179.

De Vuyst, H., Steyaert, S., Van Renterghem, L., Claeys, P., Muchiri, L., Sitati, S., Vansteelandt, S., Quint, W., Kleter, B., Van Marck, E., and Temmerman, M. (2003). Distribution of human papillomavirus in a family planning population in Nairobi, Kenya. *Sex Transm. Dis.* **30**(2), 137–142.

Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998). A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **72**(10), 8309–8315.

Djerbi, M., Screpanti, V., Catrina, A. I., Bogen, B., Biberfeld, P., and Grandien, A. (1999). The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J. Exp. Med.* **190**(7), 1025–1032.

Dourmishev, L. A., Dourmishev, A. L., Palmeri, D., Schwartz, R. A., and Lukac, D. M. (2003). Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67**(2), 175–212, table of contents.

Drexler, H. G., Uphoff, C. C., Gaidano, G., and Carbone, A. (1998). Lymphoma cell lines: *In vitro* models for the study of HHV-8+ primary effusion lymphomas (body cavity-based lymphomas). *Leukemia* **12**(10), 1507–1517.

Dunne, E. F., Nielson, C. M., Stone, K. M., Markowitz, L. E., and Giuliano, A. R. (2006). Prevalence of HPV infection among men: A systematic review of the literature. *J. Infect. Dis.* **194**(8), 1044–1057.

Durack, D. T. (1981). Opportunistic infections and Kaposi's sarcoma in homosexual men. *N. Engl. J. Med.* **305**(24), 1465–1467.

Durante, A. J., Williams, A. B., Da Costa, M., Darragh, T. M., Khoshnood, K., and Palefsky, J. M. (2003). Incidence of anal cytological abnormalities in a cohort of human immunodeficiency virus-infected women. *Cancer Epidemiol. Biomark. Prev.* **12** (7), 638–642.

Durst, M., Gissmann, L., Ikenberg, H., and zur Hausen, H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA* **80**(12), 3812–3815.

Durst, M., Croce, C. M., Gissmann, L., Schwarz, E., and Huebner, K. (1987). Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proc. Natl. Acad. Sci. USA* **84**(4), 1070–1074.

Eichler, A. F., and Batchelor, T. T. (2006). Primary central nervous system lymphoma: Presentation, diagnosis and staging. *Neurosurg. Focus* **21**(5), E15.

El-Mofty, S. K., and Lu, D. W. (2003). Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: A distinct clinicopathologic and molecular disease entity. *Am. J. Surg. Pathol.* **27**(11), 1463–1470.

Eliopoulos, A. G., and Young, L. S. (1998). Activation of the cJun N-terminal kinase (JNK) pathway by the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1). *Oncogene* **16**, 1731–1742.

Eliopoulos, A. G., Blake, S. M., Floettmann, J. E., Rowe, M., and Young, L. S. (1999). Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. *J. Virol.* **73**, 1023–1035.

Epeldegui, M., Widney, D. P., and Martinez-Maza, O. (2006). Pathogenesis of AIDS lymphoma: Role of oncogenic viruses and B cell activation-associated molecular lesions. *Curr. Opin. Oncol.* **18**(5), 444–448.

Fahraeus, R., Rymo, L., Rhim, J. S., and Klein, G. (1990). Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. *Nature* **345**, 447–449.

Fakhry, C., and Gillison, M. L. (2006). Clinical implications of human papillomavirus in head and neck cancers. *J. Clin. Oncol.* **24**(17), 2606–2611.

Fan, W., Bubman, D., Chadburn, A., Harrington, W. J., Jr., Cesarman, E., and Knowles, D. M. (2005). Distinct subsets of primary effusion lymphoma can be identified based on their cellular gene expression profile and viral association. *J. Virol.* **79**(2), 1244–1251.

Fassone, L., Bhatia, K., Gutierrez, M., Capello, D., Gloghini, A., Dolcetti, R., Vivenza, D., Ascoli, V., Lo Coco, F., Pagani, L., Dotti, G., Rambaldi, A., *et al.* (2000). Molecular profile of Epstein-Barr virus infection in HHV-8-positive primary effusion lymphoma. *Leukemia* **14**(2), 271–277.

Floettmann, J. E., and Rowe, M. (1997). Epstein-Barr virus latent membrane protein-1 (LMP1) C-terminus activation region 2 (CTAR2) maps to the far C-terminus and requires oligomerization for NF-κB activation. *Oncogene* **15**, 1851–1858.

Flore, O., Rafii, S., Ely, S., O'Leary, J. J., Hyjek, E. M., and Cesarman, E. (1998). Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Nature* **394**(6693), 588–592.

Flores, E. R., and Lambert, P. F. (1997). Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J. Virol.* **71**(10), 7167–7179.

Foglieni, C., Scabini, S., Belloni, D., Broccolo, F., Lusso, P., Malnati, M. S., and Ferrero, E. (2005). Productive infection of HUVEC by HHV-8 is associated with changes compatible with angiogenic transformations. *Eur. J. Histochem.* **49**(3), 273–284.

Frattini, M. G., and Laimins, L. A. (1994). Binding of the human papillomavirus E1 origin-recognition protein is regulated through complex formation with the E2 enhancer-binding protein. *Proc. Natl. Acad. Sci. USA* **91**(26), 12398–12402.

Freitas, R. B., Freitas, M. R., and Linhares, A. C. (2002). Prevalence of human herpesvirus 8 antibodies in the population of Belem, Para, Brazil. *Rev. Inst. Med. Trop. Sao Paulo* **44**(6), 309–313.

Friborg, J., Jr., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* **402**(6764), 889–894.

Friedman-Kien, A. E., and Saltzman, B. R. (1990). Clinical manifestations of classical, endemic African, and epidemic AIDS-associated Kaposi's sarcoma. *J. Am. Acad. Dermatol.* **22**(6 Pt 2), 1237–1250.

Fries, K. L., Miller, W. E., and Raab-Traub, N. (1996). Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. *J. Virol.* **70**, 8653–8659.

Frisch, M., Biggar, R. J., and Goedert, J. J. (2000). Human papillomavirus-associated cancers in patients with human immunodeficiency virus infection and acquired immunodeficiency syndrome. *J. Natl. Cancer Inst.* **92**(18), 1500–1510.

Fujimuro, M., Wu, F. Y., ApRhys, C., Kajumbula, H., Young, D. B., Hayward, G. S., and Hayward, S. D. (2003). A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat. Med.* **9**(3), 300–306.

Gambus, G., Bourboulia, D., Esteve, A., Lahoz, R., Rodriguez, C., Bolao, F., Sirera, G., Muga, R., del Romero, J., Boshoff, C., Whitby, D., and Casabona, J. (2001). Prevalence and distribution of HHV-8 in different subpopulations, with and without HIV infection, in Spain. *AIDS* **15**(9), 1167–1174.

Gange, R. W., and Jones, E. W. (1978). Kaposi's sarcoma and immunosuppressive therapy: An appraisal. *Clin. Exp. Dermatol.* **3**(2), 135–146.

Gao, S. J., Kingsley, L., Hoover, D. R., Spira, T. J., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Parry, P., Chang, Y., and Moore, P. S. (1996a). Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. *N. Engl. J. Med.* **335**(4), 233–241.

Gao, S. J., Kingsley, L., Li, M., Zheng, W., Parravicini, C., Ziegler, J., Newton, R., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Chang, Y., and Moore, P. S. (1996b). KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. *Nat. Med.* **2**(8), 925–928.

Gates, A. E., and Kaplan, L. D. (2002). AIDS malignancies in the era of highly active antiretroviral therapy. *Oncology (Williston Park, N.Y.)* **16**(5), 657–665 discussion 665, 668–670.

Gershengorn, M. C., Geras-Raaka, E., Varma, A., and Clark-Lewis, I. (1998). Chemokines activate Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor in mammalian cells in culture. *J. Clin. Invest.* **102**(8), 1469–1472.

Gillison, M. L., Koch, W. M., Capone, R. B., Spafford, M., Westra, W. H., Wu, L., Zahurak, M. L., Daniel, R. W., Viglione, M., Symer, D. E., Shah, K. V., and Sidransky, D. (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J. Natl. Cancer Inst.* **92**(9), 709–720.

Giraldo, G., Beth, E., and Huang, E. S. (1980). Kaposi's sarcoma and its relationship to cytomegalovirus (CMNV). III. CMV DNA and CMV early antigens in Kaposi's sarcoma. *Int. J. Cancer* **26**(1), 23–29.

Gires, O., Zimber-Strobl, U., Gonnella, R., Ueffing, M., Marschall, G., Zeidler, R., Pich, D., and Hammerschmidt, W. (1997). Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. *EMBO J.* **16**, 6131–6140.

Gires, O., Kohlhuber, F., Kilger, E., Baumann, M., Kieser, A., Kaiser, C., Zeidler, R., Scheffer, B., Ueffing, M., and Hammerschmidt, W. (1999). Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J.* **18**, 3064–3073.

Gissmann, L., Boshart, M., Durst, M., Ikenberg, H., Wagner, D., and zur Hausen, H. (1984). Presence of human papillomavirus in genital tumors. *J. Invest. Dermatol.* **83**(Suppl. 1), 26S–28S.

Giuliano, A. R., Papenfuss, M., Schneider, A., Nour, M., and Hatch, K. (1999). Risk factors for high-risk type human papillomavirus infection among Mexican-American women. *Cancer Epidemiol. Biomark. Prev.* **8**(7), 615–620.

Gloeckler Ries, L. A., Reichman, M. E., Lewis, D. R., Hankey, B. F., and Edwards, B. K. (2003). Cancer survival and incidence from the Surveillance, Epidemiology, and End Results (SEER) program. *Oncologist* **8**(6), 541–552.

Goedert, J. J. (2000). The epidemiology of acquired immunodeficiency syndrome malignancies. *Semin. Oncol.* **27**(4), 390–401.

Goedert, J. J., Cote, T. R., Virgo, P., Scoppa, S. M., Kingma, D. W., Gail, M. H., Jaffe, E. S., and Biggar, R. J. (1998). Spectrum of AIDS-associated malignant disorders. *Lancet* **351**(9119), 1833–1839.

Gravitt, P. E., Kamath, A. M., Gaffikin, L., Chirenje, Z. M., Womack, S., and Shah, K. V. (2002). Human papillomavirus genotype prevalence in high-grade squamous intraepithelial lesions and colposcopically normal women from Zimbabwe. *Int. J. Cancer* **100**(6), 729–732.

Grossmann, C., Podgrabinska, S., Skobe, M., and Ganem, D. (2006). Activation of NF-kappaB by the latent vFLIP gene of Kaposi's sarcoma-associated herpesvirus is required for the spindle shape of virus-infected endothelial cells and contributes to their proinflammatory phenotype. *J. Virol.* **80**(14), 7179–7185.

Grulich, A. E., and Vajdic, C. M. (2005). The epidemiology of non-Hodgkin lymphoma. *Pathology* **37**(6), 409–419.

Grulich, A. E., Li, Y., McDonald, A., Correll, P. K., Law, M. G., and Kaldor, J. M. (2002). Rates of non-AIDS-defining cancers in people with HIV infection before and after AIDS diagnosis. *AIDS* **16**(8), 1155–1161.

Guasparri, I., Keller, S. A., and Cesarman, E. (2004). KSHV vFLIP is essential for the survival of infected lymphoma cells. *J. Exp. Med.* **199**(7), 993–1003.

Hankey, B. F., Ries, L. A., and Edwards, B. K. (1999). The surveillance, epidemiology, and end results program: A national resource. *Cancer Epidemiol. Biomark. Prev.* **8**(12), 1117–1121.

Harwood, A. R., Osoba, D., Hofstader, S. L., Goldstein, M. B., Cardella, C. J., Holecek, M. J., Kunynetz, R., and Giammarco, R. A. (1979). Kaposi's sarcoma in recipients of renal transplants. *Am. J. Med.* **67**(5), 759–765.

Hassen, E., Chaieb, A., Letaief, M., Khairi, H., Zakhama, A., Remadi, S., and Chouchane, L. (2003). Cervical human papillomavirus infection in Tunisian women. *Infection* **31**(3), 143–148.

Hawes, S. E., Critchlow, C. W., Faye Niang, M. A., Diouf, M. B., Diop, A., Toure, P., Aziz Kasse, A., Dembele, B., Salif Sow, P., Coll-Seck, A. M., Kuypers, J. M., and Kiviat, N. B. (2003). Increased risk of high-grade cervical squamous intraepithelial lesions and invasive cervical cancer among African women with human immunodeficiency virus type 1 and 2 infections. *J. Infect. Dis.* **188**(4), 555–563.

Heard, I., Palefsky, J. M., and Kazatchkine, M. D. (2004). The impact of HIV antiviral therapy on human papillomavirus (HPV) infections and HPV-related diseases. *Antivir. Ther.* **9**(1), 13–22.

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E., and Rickinson, A. (1991). Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* **65**, 1107–1115.

Hengge, U. R., Ruzicka, T., Tyring, S. K., Stuschke, M., Roggendorf, M., Schwartz, R. A., and Seeber, S. (2002). Update on Kaposi's sarcoma and other HHV8 associated diseases. Part 1: Epidemiology, environmental predispositions, clinical manifestations, and therapy. *Lancet Infect. Dis.* **2**(5), 281–292.

Higuchi, M., Kieff, E., and Izumi, K. M. (2002). The Epstein-Barr virus latent membrane protein 1 putative Janus kinase 3 (JAK3) binding domain does not mediate JAK3 association or activation in B-lymphoma or lymphoblastoid cell lines. *J. Virol.* **76**(1), 455–459.

Hoffmann, C., Tabrizian, S., Wolf, E., Eggers, C., Stoehr, A., Plettenberg, A., Buhk, T., Stellbrink, H. J., Horst, H. A., Jager, H., and Rosenkranz, T. (2001). Survival of AIDS patients with primary central nervous system lymphoma is dramatically improved by HAART-induced immune recovery. *AIDS* **15**(16), 2119–2127.

Hoffmann, R., Hirt, B., Bechtold, V., Beard, P., and Raj, K. (2006). Different modes of human papillomavirus DNA replication during maintenance. *J. Virol.* **80**(9), 4431–4439.

Horenstein, M. G., Nador, R. G., Chadburn, A., Hyjek, E. M., Inghirami, G., Knowles, D. M., and Cesarman, E. (1997). Epstein-Barr virus latent gene expression in primary effusion lymphomas containing Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. *Blood* **90**(3), 1186–1191.

Howley, P. M. (1996). Papillomavirinae: The viruses and their replication. *In* "Virology," 2nd edn., pp. 2045–2076. Lippincott-Raven Publishers, Philadelphia, PA.

Howley, P. M., Munger, K., Werness, B. A., Phelps, W. C., and Schlegel, R. (1989). Molecular mechanisms of transformation by the human papillomaviruses. *Princess Takamatsu Symp.* **20**, 199–206.

Huen, D. S., Henderson, S. A., Croom-Carter, S., and Rowe, M. (1995). The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-κB and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* **10**, 549–560.

Iscovich, J., Boffetta, P., Franceschi, S., Azizi, E., and Sarid, R. (2000). Classic kaposi sarcoma: Epidemiology and risk factors. *Cancer* **88**(3), 500–517.

Ivers, L. C., Kim, A. Y., and Sax, P. E. (2004). Predictive value of polymerase chain reaction of cerebrospinal fluid for detection of Epstein-Barr virus to establish the diagnosis of HIV-related primary central nervous system lymphoma. *Clin. Infect. Dis.* **38**(11), 1629–1632.

Izumi, K., and Kieff, E. (1997). The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B-lymphocyte growth transformation and activate NF-κB. *Proc. Natl. Acad. Sci.* **94**, 12592–12597.

Izumi, K. M., Kaye, K. M., and Kieff, E. D. (1997). The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. *Proc. Natl. Acad. Sci. USA* **94**, 1447–1452.

Izumi, K. M., McFarland, E. C., Ting, A. T., Riley, E. A., Seed, B., and Kieff, E. D. (1999). The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappaB activation. *Mol. Cell Biol.* **19**, 5759–5767.

Jastreboff, A. M., and Cymet, T. (2002). Role of the human papilloma virus in the development of cervical intraepithelial neoplasia and malignancy. *Postgrad. Med. J.* **78**(918), 225–228.

Jeon, S., Allen-Hoffmann, B. L., and Lambert, P. F. (1995). Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J. Virol.* **69**(5), 2989–2997.

Jordan, L. B., and Monaghan, H. (2004). Pathology of the cervix: Recent developments. *Clin. Oncol. (R. Coll. Radiol.)* **16**(4), 248–254.

Kapadia, S. B., and Krause, J. R. (1977). Kaposi's sarcoma after long-term alkylating agent therapy for multiple myeloma. *South Med. J.* **70**(8), 1011–1013.

Kaplan, L. D., Kahn, J. O., Crowe, S., Northfelt, D., Neville, P., Grossberg, H., Abrams, D. I., Tracey, J., Mills, J., and Volberding, P. A. (1991). Clinical and virologic effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients receiving chemotherapy for human immunodeficiency virus-associated non-Hodgkin's lymphoma: Results of a randomized trial. *J. Clin. Oncol.* **9**(6), 929–940.

Kaplan, L. D., Straus, D. J., Testa, M. A., Von Roenn, J., Dezube, B. J., Cooley, T. P., Herndier, B., Northfelt, D. W., Huang, J., Tulpule, A., and Levine, A. M. (1997). Low-dose compared with standard-dose m-BACOD chemotherapy for non-Hodgkin's lymphoma associated with human immunodeficiency virus infection. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group. *N. Engl. J. Med.* **336**(23), 1641–1648.

Kaposi, M. (1872). Idiopathic multiple pigmented sarcoma of the skin. *Arch. Dermatol. Syphilis* **4**, 265–273.

Katano, H., Sato, Y., Kurata, T., Mori, S., and Sata, T. (2000). Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. *Virology* **269**(2), 335–344.

Kawana, Y., Kawana, K., Yoshikawa, H., Taketani, Y., Yoshiike, K., and Kanda, T. (2001). Human papillomavirus type 16 minor capsid protein l2 N-terminal region containing a common neutralization epitope binds to the cell surface and enters the cytoplasm. *J. Virol.* **75**(5), 2331–2336.

Kaye, K. M., Izumi, K. M., and Kieff, E. (1993). Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl. Acad. Sci. USA* **90**(19), 9150–9154.

Kaye, K., Devergne, O., Harada, J., Izumi, K., Yalamanchili, R., Kieff, E., and Mosialos, G. (1996). Tumor necrosis factor receptor associated factor 2 is a mediator of NF-κB activation by latent infection membrane protein 1, the Epstein-Barr virus transforming protein. *Proc. Natl. Acad. Sci. USA* **93**, 11085–11090.

Kazanji, M., Dussart, P., Duprez, R., Tortevoye, P., Pouliquen, J. F., Vandekerkhove, J., Couppie, P., Morvan, J., Talarmin, A., and Gessain, A. (2005). Serological and molecular evidence that human herpesvirus 8 is endemic among Amerindians in French Guiana. *J. Infect. Dis.* **192**(9), 1525–1529.

Kelekar, A., and Cole, M. D. (1986). Tumorigenicity of fibroblast lines expressing the adenovirus E1a, cellular p53, or normal c-myc genes. *Mol. Cell Biol.* **6**(1), 7–14.

Kieff, E. (1996). Epstein-Barr virus and its replication. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 3rd edn., pp. 2343–2396. Lippinscott-Raven Publishers, Philadelphia, PA.

Kilger, E., Kieser, A., Baumann, M., and Hammerschmidt, W. (1998). Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* **17**, 1700–1709.

Kirk, O., Pedersen, C., Cozzi-Lepri, A., Antunes, F., Miller, V., Gatell, J. M., Katlama, C., Lazzarin, A., Skinhoj, P., and Barton, S. E. (2001). Non-Hodgkin lymphoma in HIV-infected patients in the era of highly active antiretroviral therapy. *Blood* **98**(12), 3406–3412.

Klaskala, W., Brayfield, B. P., Kankasa, C., Bhat, G., West, J. T., Mitchell, C. D., and Wood, C. (2005). Epidemiological characteristics of human herpesvirus-8 infection in a large population of antenatal women in Zambia. *J. Med. Virol.* **75**(1), 93–100.

Klencke, B. J., and Palefsky, J. M. (2003). Anal cancer: An HIV-associated cancer. *Hematol. Oncol. Clin. North Am.* **17**(3), 859–872.

Klepp, O., Dahl, O., and Stenwig, J. T. (1978). Association of Kaposi's sarcoma and prior immunosuppressive therapy: A 5-year material of Kaposi's sarcoma in Norway. *Cancer* **42**(6), 2626–2630.

Kliche, S., Nagel, W., Kremmer, E., Atzler, C., Ege, A., Knorr, T., Koszinowski, U., Kolanus, W., and Haas, J. (2001). Signaling by human herpesvirus 8 kaposin A through direct membrane recruitment of cytohesin-1. *Mol. Cell* **7**(4), 833–843.

Knowles, D. M. (2003). Etiology and pathogenesis of AIDS-related non-Hodgkin's lymphoma. *Hematol. Oncol. Clin. North Am.* **17**(3), 785–820.

Kohl, N. E., and Ruley, H. E. (1987). Role of c-myc in the transformation of REF52 cells by viral and cellular oncogenes. *Oncogene* **2**(1), 41–48.

Koutsky, L. (1997). Epidemiology of genital human papillomavirus infection. *Am. J. Med.* **102**(5A), 3–8.

Krithivas, A., Young, D. B., Liao, G., Greene, D., and Hayward, S. D. (2000). Human herpesvirus 8 LANA interacts with proteins of the mSin3 corepressor complex and negatively regulates Epstein-Barr virus gene expression in dually infected PEL cells. *J. Virol.* **74**(20), 9637–9645.

Kuo, S. R., Liu, J. S., Broker, T. R., and Chow, L. T. (1994). Cell-free replication of the human papillomavirus DNA with homologous viral E1 and E2 proteins and human cell extracts. *J. Biol. Chem.* **269**(39), 24058–24065.

Lacoste, V., Judde, J. G., Bestett, G., Cadranel, J., Antoine, M., Valensi, F., Delabesse, E., Macintyre, E., and Gessain, A. (2000). Virological and molecular characterisation of a new B lymphoid cell line, established from an AIDS patient with primary effusion lymphoma, harbouring both KSHV/HHV8 and EBV viruses. *Leuk. Lymphoma* **38**(3–4), 401–409.

Lagunoff, M., and Ganem, D. (1997). The structure and coding organization of the genomic termini of Kaposi's sarcoma-associated herpesvirus. *Virology* **236**(1), 147–154.

Lagunoff, M., Majeti, R., Weiss, A., and Ganem, D. (1999). Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. USA* **96**(10), 5704–5709.

Langley, C. L., Benga-De, E., Critchlow, C. W., Ndoye, I., Mbengue-Ly, M. D., Kuypers, J., Woto-Gaye, G., Mboup, S., Bergeron, C., Holmes, K. K., and Kiviat, N. B. (1996). HIV-1, HIV-2, human papillomavirus infection and cervical neoplasia in high-risk African women. *AIDS* **10**(4), 413–417.

Laurence, J. (2003). Repetitive and consistent cervicovaginal exposure to certain viral pathogens appears to protect against their sexual acquisition in some women: Potential mechanisms. *J. Reprod. Immunol.* **58**(1), 79–91.

Lazzi, S., Ferrari, F., Nyongo, A., Palummo, N., de Milito, A., Zazzi, M., Leoncini, L., Luzi, P., and Tosi, P. (1998). HIV-associated malignant lymphomas in Kenya (Equatorial Africa). *Hum. Pathol.* **29**(11), 1285–1289.

Lee, J. K., and Hurwitz, J. (2000). Isolation and characterization of various complexes of the minichromosome maintenance proteins of Schizosaccharomyces pombe. *J. Biol. Chem.* **275**(25), 18871–18878.

Lee, H., Guo, J., Li, M., Choi, J. K., DeMaria, M., Rosenzweig, M., and Jung, J. U. (1998a). Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. *Mol. Cell Biol.* **18**(9), 5219–5228.

Lee, H., Veazey, R., Williams, K., Li, M., Guo, J., Neipel, F., Fleckenstein, B., Lackner, A., Desrosiers, R. C., and Jung, J. U. (1998b). Deregulation of cell growth by the K1 gene of Kaposi's sarcoma-associated herpesvirus. *Nat. Med.* **4**(4), 435–440.

Lee, B. S., Connole, M., Tang, Z., Harris, N. L., and Jung, J. U. (2003). Structural analysis of the Kaposi's sarcoma-associated herpesvirus K1 protein. *J. Virol.* **77**(14), 8072–8086.

Lennette, E. T., Blackbourn, D. J., and Levy, J. A. (1996). Antibodies to human herpesvirus type 8 in the general population and in Kaposi's sarcoma patients. *Lancet* **348**(9031), 858–861.

Levine, A. M. (2006). AIDS-related lymphoma. *Semin. Oncol. Nurs.* **22**(2), 80–89.

Li, J. J., Huang, Y. Q., Cockerell, C. J., and Friedman-Kien, A. E. (1996). Localization of human herpes-like virus type 8 in vascular endothelial cells and perivascular spindle-shaped cells of Kaposi's sarcoma lesions by in situ hybridization. *Am. J. Pathol.* **148**(6), 1741–1748.

Li, M., Beard, P., Estes, P. A., Lyon, M. K., and Garcea, R. L. (1998). Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. *J. Virol.* **72**(3), 2160–2167.

Liang, Y., Chang, J., Lynch, S. J., Lukac, D. M., and Ganem, D. (2002). The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. *Genes Dev.* **16**(15), 1977–1989.

Liebowitz, D., Wang, D., and Kieff, E. (1986). Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. *J. Virol.* **58**(1), 233–237.

Lillo, F. B., Ferrari, D., Veglia, F., Origoni, M., Grasso, M. A., Lodini, S., Mastrorilli, E., Taccagni, G., Lazzarin, A., and Uberti-Foppa, C. (2001). Human papillomavirus infection and associated cervical disease in human immunodeficiency virus-infected women: Effect of highly active antiretroviral therapy. *J. Infect. Dis.* **184**(5), 547–551.

Londesborough, P., Ho, L., Terry, G., Cuzick, J., Wheeler, C., and Singer, A. (1996). Human papillomavirus genotype as a predictor of persistence and development of high-grade lesions in women with minor cervical abnormalities. *Int. J. Cancer* **69**(5), 364–368.

Longnecker, R. (2000). Epstein-Barr virus latency: LMP2, a regulator or means for Epstein-Barr virus persistence? *Adv. Cancer Res.* **79**, 175–200.

Longnecker, R., Merchant, M., Brown, M. E., Fruehling, S., Bickford, J. O., Ikeda, M., and Harty, R. N. (2000). WW- and SH3-domain interactions with Epstein-Barr virus LMP2A. *Exp. Cell Res.* **257**(2), 332–340.

Lopez Amado, M., Castro Lareo, I., LozanoRamirez, A., and Caballero Torcuato, L. (1996). Epidemiology of otorhinolaryngological squamous cell papillomas in a Spanish subpopulation during a 20 year period. *Rev. Laryngol. Otol. Rhinol. (Bord)* **117**(2), 105–110.

Lothe, F., and Murray, J. F. (1962). Kaposi's sarcoma: Autopsy findings in the African. *Acta Unio Int. Contra Cancrum* **18**, 429–452.

Mansky, K. C., Batiza, A., and Lambert, P. F. (1997). Bovine papillomavirus type 1 E1 and simian virus 40 large T antigen share regions of sequence similarity required for multiple functions. *J. Virol.* **71**(10), 7600–7608.

Mao, C., Koutsky, L. A., Ault, K. A., Wheeler, C. M., Brown, D. R., Wiley, D. J., Alvarez, F. B., Bautista, O. M., Jansen, K. U., and Barr, E. (2006). Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: A randomized controlled trial. *Obstet. Gynecol.* **107**(1), 18–27.

Massad, L. S., Schneider, M. F., Watts, D. H., Strickler, H. D., Melnick, S., Palefsky, J., Anastos, K., Levine, A. M., and Minkoff, H. (2004). HPV testing for triage of HIV-infected women with papanicolaou smears read as atypical squamous cells of uncertain significance. *J. Womens Health (Larchmt)* **13**(2), 147–153.

Mayama, S., Cuevas, L. E., Sheldon, J., Omar, O. H., Smith, D. H., Okong, P., Silvel, B., Hart, C. A., and Schulz, T. F. (1998). Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. *Int. J. Cancer* **77**(6), 817–820.

Mayaud, P., Gill, D. K., Weiss, H. A., Uledi, E., Kopwe, L., Todd, J., ka-Gina, G., Grosskurth, H., Hayes, R. J., Mabey, D. C., and Lacey, C. J. (2001). The interrelation

of HIV, cervical human papillomavirus, and neoplasia among antenatal clinic attenders in Tanzania. *Sex Transm. Infect.* **77**(4), 248–254.

Mbulaiteye, S. M., Biggar, R. J., Goedert, J. J., and Engels, E. A. (2002). Pleural and peritoneal lymphoma among people with AIDS in the United States. *J. Acquir. Immune Defic. Syndr.* **29**(4), 418–421.

Mbulaiteye, S. M., Pfeiffer, R. M., Whitby, D., Brubaker, G. R., Shao, J., and Biggar, R. J. (2003). Human herpesvirus 8 infection within families in rural Tanzania. *J. Infect. Dis.* **187**(11), 1780–1785.

McCormick, C., and Ganem, D. (2006). Phosphorylation and function of the kaposin B direct repeats of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **80**(12), 6165–6170.

McGowan, J. P., and Shah, S. (1998). Long-term remission of AIDS-related primary central nervous system lymphoma associated with highly active antiretroviral therapy. *AIDS* **12**(8), 952–954.

Melbye, M., Cook, P. M., Hjalgrim, H., Begtrup, K., Simpson, G. R., Biggar, R. J., Ebbesen, P., and Schulz, T. F. (1998). Risk factors for Kaposi's-sarcoma-associated herpesvirus (KSHV/HHV-8) seropositivity in a cohort of homosexual men, 1981–1996. *Int. J. Cancer* **77**(4), 543–548.

Memar, O. M., Rady, P. L., and Tyring, S. K. (1995). Human herpesvirus-8: Detection of novel herpesvirus-like DNA sequences in Kaposi's sarcoma and other lesions. *J. Mol. Med.* **73**(12), 603–609.

Milde-Langosch, K. M., Loning, T., Meichsner, M., and Henke, R. P. (1989). (HPV infections in tumors of the upper respiratory and digestive tract. In situ hybridization and dot-blot hybridization with biotinylated DNA probes). *Acta Histochem. Suppl.* **37**, 103–108.

Miller, W. E., Earp, H. S., and Raab-Traub, N. (1995). The Epstein-Barr virus latent membrane protein1 induces expression of the epidermal growth factor receptor. *J. Virol.* **69**, 4390–4398.

Miller, W. E., Mosialos, G., Kieff, E., and Raab-Traub, N. (1997). Epstein-Barr virus LMP1 induction of the EGFR is mediated through a TRAF signaling pathway distinct from NF-κB activation. *J. Virol.* **71**, 586–594.

Miller, W., Cheshire, J., and Raab-Traub, N. (1998). Interaction of tumor necrosis factor receptor-associated factor signaling proteins with the latent membrane protein 1 PXQXT motif is essential for induction of epidermal growth factor receptor expression. *Mol. Cell Biol.* **18**, 2835–2844.

Minchiotti, S., Masucci, L., Serapiao Dos Santos, M., Perrella, E., Graffeo, R., Lambiase, A., and Bonini, S. (2006). Conjunctival papilloma and human papillomavirus: Identification of HPV types by PCR. *Eur. J. Ophthalmol.* **16**(3), 473–477.

Mincione, G. P., Taddei, G. L., Wolovsky, M., Calzolari, A., and Mincione, F. (1992). Detection of human papillomavirus (HPV) DNA type 6/11 in a conjunctival papilloma by in situ hybridization with biotinylated probes. *Pathologica* **84**(1092), 483–488.

Mineta, H., Ogino, T., Amano, H. M., Ohkawa, Y., Araki, K., Takebayashi, S., and Miura, K. (1998). Human papilloma virus (HPV) type 16 and 18 detected in head and neck squamous cell carcinoma. *Anticancer Res.* **18**(6B), 4765–4768.

Minkoff, H., Ahdieh, L., Massad, L. S., Anastos, K., Watts, D. H., Melnick, S., Muderspach, L., Burk, R., and Palefsky, J. (2001). The effect of highly active antiretroviral therapy on cervical cytologic changes associated with oncogenic HPV among HIV-infected women. *AIDS* **15**(16), 2157–2164.

Mitchell, T., and Sugden, B. (1995). Stimulation of NF-κB-mediated transcription by mutant derivatives of the latent membrane protein of Epstein-Barr virus. *J. Virol.* **69**(5), 2968–2976.

Molden, J., Chang, Y., You, Y., Moore, P. S., and Goldsmith, M. A. (1997). A Kaposi's sarcoma-associated herpesvirus-encoded cytokine homolog (vIL-6) activates signaling through the shared gp130 receptor subunit. *J. Biol. Chem.* **272**(31), 19625–19631.

Montaner, S., Sodhi, A., Molinolo, A., Bugge, T. H., Sawai, E. T., He, Y., Li, Y., Ray, P. E., and Gutkind, J. S. (2003). Endothelial infection with KSHV genes *in vivo* reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. *Cancer Cell* **3**(1), 23–36.

Moore, P., and Chang, Y. (2001). Kaposi's sacrcoma-associated herpesvirus. *In* "Virology" (D. M. Knipe, Ed.), pp. 2803–2833. Lippincott Williams & Wilkins, Philadelphia, PA.

Moore, P. S., Boshoff, C., Weiss, R. A., and Chang, Y. (1996a). Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* **274**(5293), 1739–1744.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996b). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae. *J. Virol.* **70**(1), 549–558.

Moore, P. S., Kingsley, L. A., Holmberg, S. D., Spira, T., Gupta, P., Hoover, D. R., Parry, J. P., Conley, L. J., Jaffe, H. W., and Chang, Y. (1996c). Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. *AIDS* **10**(2), 175–180.

Morison, L. (2001). The global epidemiology of HIV/AIDS. *Br. Med. Bull.* **58**, 7–18.

Moses, A. V., Fish, K. N., Ruhl, R., Smith, P. P., Strussenberg, J. G., Zhu, L., Chandran, B., and Nelson, J. A. (1999). Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. *J. Virol.* **73**(8), 6892–6902.

Mosunjac, M. B., Tadros, T., Beach, R., and Majmudar, B. (2003). Cervical schistosomiasis, human papilloma virus (HPV), and human immunodeficiency virus (HIV): A dangerous coexistence or coincidence? *Gynecol. Oncol.* **90**(1), 211–214.

Motti, P. G., Dallabetta, G. A., Daniel, R. W., Canner, J. K., Chiphangwi, J. D., Liomba, G. N., Yang, L., and Shah, K. V. (1996). Cervical abnormalities, human papillomavirus, and human immunodeficiency virus infections in women in Malawi. *J. Infect. Dis.* **173**(3), 714–717.

Mueller, N. (1999). Overview of the epidemiology of malignancy in immune deficiency. *J. Acquir. Immune Defic. Syndr.* **21**(Suppl. 1), S5–S10.

Munger, K., Phelps, W. C., Bubb, V., Howley, P. M., and Schlegel, R. (1989). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* **63**(10), 4417–4421.

Munoz, N., Bosch, F. X., Castellsague, X., Diaz, M., de Sanjose, S., Hammouda, D., Shah, K. V., and Meijer, C. J. (2004). Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int. J. Cancer* **111**(2), 278–285.

Muralidhar, S., Pumfery, A. M., Hassani, M., Sadaie, M. R., Kishishita, M., Brady, J. N., Doniger, J., Medveczky, P., and Rosenthal, L. J. (1998). Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. *J. Virol.* **72**(6), 4980–4988.

Muralidhar, S., Veytsmann, G., Chandran, B., Ablashi, D., Doniger, J., and Rosenthal, L. J. (2000). Characterization of the human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) oncogene, kaposin (ORF K12). *J. Clin. Virol.* **16**(3), 203–213.

Nador, R. G., Cesarman, E., Chadburn, A., Dawson, D. B., Ansari, M. Q., Sald, J., and Knowles, D. M. (1996). Primary effusion lymphoma: A distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood* **88**(2), 645–656.

Nakano, K. (1994). Characteristics of human papillomavirus (HPV) infection in papilloma of the head and neck—detection of HPV according to clinical features and type specificity in the head and neck. *Nippon Jibiinkoka Gakkai Kaiho* **97**(8), 1381–1392.

Nakano, K., Isegawa, Y., Zou, P., Tadagaki, K., Inagi, R., and Yamanishi, K. (2003). Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded vMIP-I and vMIP-II induce signal transduction and chemotaxis in monocytic cells. *Arch. Virol.* **148**(5), 871–890.

Navarro, W. H., and Kaplan, L. D. (2006). AIDS-related lymphoproliferative disease. *Blood* **107**(1), 13–20.

Newell, M. E., Hoy, J. F., Cooper, S. G., DeGraaff, B., Grulich, A. E., Bryant, M., Millar, J. L., Brew, B. J., and Quinn, D. I. (2004). Human immunodeficiency virus-related primary central nervous system lymphoma: Factors influencing survival in 111 patients. *Cancer* **100**(12), 2627–2636.

Newton, R., Ziegler, J., Ateenyi-Agaba, C., Bousarghin, L., Casabonne, D., Beral, V., Mbidde, E., Carpenter, L., Reeves, G., Parkin, D. M., Wabinga, H., Mbulaiteye, S., *et al.* (2002). The epidemiology of conjunctival squamous cell carcinoma in Uganda. *Br. J. Cancer* **87**(3), 301–308.

Nicholas, J., Ruvolo, V. R., Burns, W. H., Sandford, G., Wan, X., Ciufo, D., Hendrickson, S. B., Guo, H. G., Hayward, G. S., and Reitz, M. S. (1997). Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. *Nat. Med.* **3**(3), 287–292.

Noy, A. (2006). Update in HIV lymphoma. *Curr. Opin. Oncol.* **18**(5), 449–455.

Nzila, N., Laga, M., Thiam, M. A., Mayimona, K., Edidi, B., Van Dyck, E., Behets, F., Hassig, S., Nelson, A., Mokwa, K., *et al.* (1991). HIV and other sexually transmitted diseases among female prostitutes in Kinshasa. *AIDS* **5**(6), 715–721.

O'Farrell, N., Hoosen, A. A., Kharsany, A. B., and van den Ende, J. (1989). Sexually transmitted pathogens in pregnant women in a rural South African community. *Genitourin Med.* **65**(4), 276–280.

Oettle, A. G. (1962). Geographical and racial differences in the frequency of Kaposi's sarcoma as evidence of environmental or genetic causes. *Acta Unio Int. Contra Cancrum* **18**, 330–363.

Olsen, S. J., Chang, Y., Moore, P. S., Biggar, R. J., and Melbye, M. (1998). Increasing Kaposi's sarcoma-associated herpesvirus seroprevalence with age in a highly Kaposi's sarcoma endemic region, Zambia in 1985. *AIDS* **12**(14), 1921–1925.

Ong, C. K., Chan, S. Y., Campo, M. S., Fujinaga, K., Mavromara-Nazos, P., Labropoulou, V., Pfister, H., Tay, S. K., ter Meulen, J., and Villa, L. L. (1993). Evolution of human papillomavirus type 18: An ancient phylogenetic root in Africa and intratype diversity reflect coevolution with human ethnic groups. *J. Virol.* **67**(11), 6424–6431.

Pagano, J. S. (1999). Epstein-Barr virus: The first human tumor virus and its role in cancer. *Proc. Assoc. Am. Physicians* **111**, 573–580.

Palefsky, J. (1991). Human papillomavirus infection among HIV-infected individuals. Implications for development of malignant tumors. *Hematol. Oncol. Clin. North Am.* **5**(2), 357–370.

Palefsky, J. M. (2003). Cervical human papillomavirus infection and cervical intraepithelial neoplasia in women positive for human immunodeficiency virus in the era of highly active antiretroviral therapy. *Curr. Opin. Oncol.* **15**(5), 382–388.

Palefsky, J. M., and Barrasso, R. (1996). HPV infection and disease in men. *Obstet. Gynecol. Clin. North Am.* **23**(4), 895–916.

Pantanowitz, L., Schlecht, H. P., and Dezube, B. J. (2006). The growing problem of non-AIDS-defining malignancies in HIV. *Curr. Opin. Oncol.* **18**(5), 469–478.

Parkin, D. M., Garcia-Giannoli, H., Raphael, M., Martin, A., Katangole-Mbidde, E., Wabinga, H., and Ziegler, J. (2000). Non-Hodgkin lymphoma in Uganda: A case-control study. *AIDS* **14**(18), 2929–2936.

Patil, P., Elem, B., and Zumla, A. (1995). Pattern of adult malignancies in Zambia (1980–1989) in light of the human immunodeficiency virus type 1 epidemic. *J. Trop. Med. Hyg.* **98**(4), 281–284.

Pellett, P. E., Wright, D. J., Engels, E. A., Ablashi, D. V., Dollard, S. C., Forghani, B., Glynn, S. A., Goedert, J. J., Jenkins, F. J., Lee, T. H., Neipel, F., Todd, D. S., *et al.* (2003). Multicenter comparison of serologic assays and estimation of human herpesvirus 8 seroprevalence among US blood donors. *Transfusion* **43**(9), 1260–1268.

Penn, I. (1979). Kaposi's sarcoma in organ transplant recipients: Report of 20 cases. *Transplantation* **27**(1), 8–11.

Peyton, C. L., Gravitt, P. E., Hunt, W. C., Hundley, R. S., Zhao, M., Apple, R. J., and Wheeler, C. M. (2001). Determinants of genital human papillomavirus detection in a US population. *J. Infect. Dis.* **183**(11), 1554–1564.

Piketty, C., Darragh, T. M., Da Costa, M., Bruneval, P., Heard, I., Kazatchkine, M. D., and Palefsky, J. M. (2003). High prevalence of anal human papillomavirus infection and anal cancer precursors among HIV-infected persons in the absence of anal intercourse. *Ann. Intern. Med.* **138**(6), 453–459.

Piketty, C., Darragh, T. M., Heard, I., Da Costa, M., Bruneval, P., Kazatchkine, M. D., and Palefsky, J. M. (2004). High prevalence of anal squamous intraepithelial lesions in HIV-positive men despite the use of highly active antiretroviral therapy. *Sex Transm. Dis.* **31**(2), 96–99.

Plancoulaine, S., Abel, L., van Beveren, M., Tregouet, D. A., Joubert, M., Tortevoye, P., de The, G., and Gessain, A. (2000). Human herpesvirus 8 transmission from mother to child and between siblings in an endemic population. *Lancet* **356**(9235), 1062–1065.

Plancoulaine, S., Abel, L., Tregouet, D., Duprez, R., van Beveren, M., Tortevoye, P., Froment, A., and Gessain, A. (2004). Respective roles of serological status and blood specific antihuman herpesvirus 8 antibody levels in human herpesvirus 8 intrafamilial transmission in a highly endemic area. *Cancer Res.* **64**(23), 8782–8787.

Ponten, J., and Guo, Z. (1998). Precancer of the human cervix. *Cancer Surv.* **32**, 201–229.

Prakash, O., Tang, Z. Y., Peng, X., Coleman, R., Gill, J., Farr, G., and Samaniego, F. (2002). Tumorigenesis and aberrant signaling in transgenic mice expressing the human herpesvirus-8 K1 gene. *J. Natl. Cancer Inst.* **94**(12), 926–935.

Quinlivan, E. B., Zhang, C., Stewart, P. W., Komoltri, C., Davis, M. G., and Wehbie, R. S. (2002). Elevated virus loads of Kaposi's sarcoma-associated human herpesvirus 8 predict Kaposi's sarcoma disease progression, but elevated levels of human immunodeficiency virus type 1 do not. *J. Infect. Dis.* **185**(12), 1736–1744.

Rabkin, C. S., Schulz, T. F., Whitby, D., Lennette, E. T., Magpantay, L. I., Chatlynne, L., and Biggar, R. J. (1998). Interassay correlation of human herpesvirus 8 serologic tests. HHV-8 Interlaboratory Collaborative Groups. *J. Infect. Dis.* **178**(2), 304–309.

Radkov, S. A., Kellam, P., and Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat. Med.* **6**(10), 1121–1127.

Ralston, R. (1991). Complementation of transforming domains in E1a/myc chimaeras. *Nature* **353**(6347), 866–868.

Reszec, J., and Sulkowski, S. (2005). The expression of P53 protein and infection of human papilloma virus in conjunctival and eyelid neoplasms. *Int. J. Mol. Med.* **16**(4), 559–564.

Rickinson, A. B., and Kieff, E. (1996). Epstein-Barr Virus. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 3rd edn., pp. 2397–2446. Lippinscott-Raven Publishers, Philadelphia, PA.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C., and Studdert, M. J. (1992). The family herpesviridae: An update. The herpesvirus study group of the international committee on taxonomy of viruses. *Arch. Virol.* **123**(3–4), 425–449.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. USA* **93**(25), 14862–14867.

Sadler, R., Wu, L., Forghani, B., Renne, R., Zhong, W., Herndier, B., and Ganem, D. (1999). A complex translational program generates multiple novel proteins from the latently expressed kaposin (K12) locus of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **73**(7), 5722–5730.

Sakai, H., Yasugi, T., Benson, J. D., Dowhanick, J. J., and Howley, P. M. (1996). Targeted mutagenesis of the human papillomavirus type 16 E2 transactivation domain reveals

separable transcriptional activation and DNA replication functions. *J. Virol.* **70**(3), 1602–1611.

Sandberg, M., Hammerschmidt, W., and Sugden, B. (1997). Characterizarion of LMP1's association with TRAF1, TRAF2, and TRAF3. *J. Virol.* **71**, 4649–4656.

Sapp, M., Perez-Ordonez, B., Brenneman, F., Imrie, K., Morava-Protzner, I., and Lim, M. S. (2001). EBV-associated perianal Hodgkin's disease in an HIV-positive individual. *Am. J. Hematol.* **66**(1), 42–45.

Schuman, P., Ohmit, S. E., Klein, R. S., Duerr, A., Cu-Uvin, S., Jamieson, D. J., Anderson, J., and Shah, K. V. (2003). Longitudinal study of cervical squamous intraepithelial lesions in human immunodeficiency virus (HIV)-seropositive and at-risk HIV-seronegative women. *J. Infect. Dis.* **188**(1), 128–136.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A., and zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**(6006), 111–114.

Sedman, J., and Stenlund, A. (1995). Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus *in vivo* and *in vitro*. *EMBO J.* **14**(24), 6218–6228.

Seif, I. (1984). Sequence homology between the large tumor antigen of polyoma viruses and the putative E1 protein of papilloma viruses. *Virology* **138**(2), 347–352.

Serwadda, D., Wawer, M. J., Shah, K. V., Sewankambo, N. K., Daniel, R., Li, C., Lorincz, A., Meehan, M. P., Wabwire-Mangen, F., and Gray, R. H. (1999). Use of a hybrid capture assay of self-collected vaginal swabs in rural Uganda for detection of human papillomavirus. *J. Infect. Dis.* **180**(4), 1316–1319.

Shalloway, D., Johnson, P. J., Freed, E. O., Coulter, D., and Flood, W. A., Jr. (1987). Transformation of NIH 3T3 cells by cotransfection with c-src and nuclear oncogenes. *Mol. Cell Biol.* **7**(10), 3582–3590.

Shaw, K. V., and Howley, P. M. (2001). Papillomavirinae: The viruses and their replication. *In* "Fields Virology" (D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, and M. A. Martin, Eds.), 4th edn. Lippincott Williams & Wilkins, Philadelphia, PA.

Shibata, D., Weiss, L. M., Hernandez, A. M., Nathwani, B. N., Bernstein, L., and Levine, A. M. (1993). Epstein-Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus. *Blood* **81**(8), 2102–2109.

Silverberg, M. J., Ahdieh, L., Munoz, A., Anastos, K., Burk, R. D., Cu-Uvin, S., Duerr, A., Greenblatt, R. M., Klein, R. S., Massad, S., Minkoff, H., Muderspach, L., *et al.* (2002). The impact of HIV infection and immunodeficiency on human papillomavirus type 6 or 11 infection and on genital warts. *Sex Transm. Dis.* **29**(8), 427–435.

Simpson, G. R., Schulz, T. F., Whitby, D., Cook, P. M., Boshoff, C., Rainbow, L., Howard, M. R., Gao, S. J., Bohenzky, R. A., Simmonds, P., Lee, C., de Ruiter, A., *et al.* (1996). Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. *Lancet* **348**(9035), 1133–1138.

Sinclair, K. A., Woods, C. R., Kirse, D. J., and Sinal, S. H. (2005). Anogenital and respiratory tract human papillomavirus infections among children: Age, gender, and potential transmission through sexual abuse. *Pediatrics* **116**(4), 815–825.

Sirera, G., Videla, S., Pinol, M., Canadas, M. P., Llatjos, M., Ballesteros, A. L., Garcia-Cuyas, F., Castella, E., Guerola, R., Tural, C., Rey-Joly, C., and Clotet, B. (2006). High prevalence of human papillomavirus infection in the anus, penis and mouth in HIV-positive men. *AIDS* **20**(8), 1201–1204.

Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E. A., and Gutkind, J. S. (2000). The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. *Cancer Res.* **60**(17), 4873–4880.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., and Degos, L. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* **86**(4), 1276–1280.

Sparano, J. A. (2003). Human immunodeficiency virus associated lymphoma. *Curr. Opin. Oncol.* **15**(5), 372–378.

Sparano, J. A., Anand, K., Desai, J., Mitnick, R. J., Kalkut, G. E., and Hanau, L. H. (1999). Effect of highly active antiretroviral therapy on the incidence of HIV-associated malignancies at an urban medical center. *J. Acquir. Immune Defic. Syndr.* **21**(Suppl. 1), S18–S22.

St. Louis, M. E., Icenogle, J. P., Manzila, T., Kamenga, M., Ryder, R. W., Heyward, W. L., and Reeves, W. C. (Louis 1993). Genital types of papillomavirus in children of women with HIV-1 infection in Kinshasa, Zaire. *Int. J. Cancer* **54**(2), 181–184.

Stauffer, Y., Raj, K., Masternak, K., and Beard, P. (1998). Infectious human papillomavirus type 18 pseudovirions. *J. Mol. Biol.* **283**(3), 529–536.

Strickler, H. D., Palefsky, J. M., Shah, K. V., Anastos, K., Klein, R. S., Minkoff, H., Duerr, A., Massad, L. S., Celentano, D. D., Hall, C., Fazzari, M., Cu-Uvin, S., *et al.* (2003). Human papillomavirus type 16 and immune status in human immunodeficiency virus-seropositive women. *J. Natl. Cancer Inst.* **95**(14), 1062–1071.

Syrjanen, S. (2003). Human papillomavirus infections and oral tumors. *Med. Microbiol. Immunol. (Berl.)* **192**(3), 123–128.

Syrjanen, S. (2005). Human papillomavirus (HPV) in head and neck cancer. *J. Clin. Virol.* **32**(Suppl. 1), S59–S66.

Tabrizi, S. N., McCurrach, F. E., Drewe, R. H., Borg, A. J., Garland, S. M., and Taylor, H. R. (1997). Human papillomavirus in corneal and conjunctival carcinoma. *Aust. N. Z. J. Ophthalmol.* **25**(3), 211–215.

Tedeschi, R., Dillner, J., and De Paoli, P. (2002). Laboratory diagnosis of human herpesvirus 8 infection in humans. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**(12), 831–844.

ter Meulen, J., Eberhardt, H. C., Luande, J., Mgaya, H. N., Chang-Claude, J., Mtiro, H., Mhina, M., Kashaija, P., Ockert, S., Yu, X., Meinhardt, G., Gissmann, L., *et al.* (1992). Human papillomavirus (HPV) infection, HIV infection and cervical cancer in Tanzania, east Africa. *Int. J. Cancer* **51**(4), 515–521.

Thomas, J. O. (2001). Acquired immunodeficiency syndrome-associated cancers in sub-Saharan Africa. *Semin. Oncol.* **28**(2), 198–206.

Thomas, J. O., Herrero, R., Omigbodun, A. A., Ojemakinde, K., Ajayi, I. O., Fawole, A., Oladepo, O., Smith, J. S., Arslan, A., Munoz, N., Snijders, P. J., Meijer, C. J., *et al.* (2004). Prevalence of papillomavirus infection in women in Ibadan, Nigeria: A population-based study. *Br. J. Cancer* **90**(3), 638–645.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., *et al.* (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**(6624), 517–521.

Tomkowicz, B., Singh, S. P., Lai, D., Singh, A., Mahalingham, S., Joseph, J., Srivastava, S., and Srinivasan, A. (2005). Mutational analysis reveals an essential role for the LXXLL motif in the transformation function of the human herpesvirus-8 oncoprotein, kaposin. *DNA Cell Biol.* **24**(1), 10–20.

Touze, A., El Mehdaoui, S., Sizaret, P. Y., Mougin, C., Munoz, N., and Coursaget, P. (1998). The L1 major capsid protein of human papillomavirus type 16 variants affects yield of virus-like particles produced in an insect cell expression system. *J. Clin. Microbiol.* **36**(7), 2046–2051.

Trivedi, P., Takazawa, K., Zompetta, C., Cuomo, L., Anastasiadou, E., Carbone, A., Uccini, S., Belardelli, F., Takada, K., Frati, L., and Faggioni, A. (2004). Infection of HHV-8+ primary effusion lymphoma cells with a recombinant Epstein-Barr virus leads to restricted

EBV latency, altered phenotype, and increased tumorigenicity without affecting TCL1 expression. *Blood* **103**(1), 313–316.

Villa, L. L., Costa, R. L., Petta, C. A., Andrade, R. P., Ault, K. A., Giuliano, A. R., Wheeler, C. M., Koutsky, L. A., Malm, C., Lehtinen, M., Skjeldestad, F. E., Olsson, S. E., *et al.* (2005). Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: A randomised double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncol.* **6**(5), 271–278.

von Krogh, G., Wikstrom, A., Syrjanen, K., and Syrjanen, S. (1995). Anal and penile condylomas in HIV-negative and HIV-positive men: Clinical, histological and virological characteristics correlated to therapeutic outcome. *Acta Derm. Venereol.* **75**(6), 470–474.

Waddell, K. M., Lewallen, S., Lucas, S. B., Atenyi-Agaba, C., Herrington, C. S., and Liomba, G. (1996). Carcinoma of the conjunctiva and HIV infection in Uganda and Malawi. *Br. J. Ophthalmol.* **80**(6), 503–508.

Waddell, K., Magyezi, J., Bousarghin, L., Coursaget, P., Lucas, S., Downing, R., Casabonne, D., and Newton, R. (2003). Antibodies against human papillomavirus type 16 (HPV-16) and conjunctival squamous cell neoplasia in Uganda. *Br. J. Cancer* **88**(12), 2002–2003.

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and Munoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**(1), 12–19.

Wang, H. W., and Boshoff, C. (2005). Linking Kaposi virus to cancer-associated cytokines. *Trends Mol. Med.* **11**(7), 309–312.

Wang, D., Leibowitz, D., and Kieff, E. (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**, 831–840.

Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A., and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* **64**(5), 2309–2318.

Weinberg, R. A. (1985). The action of oncogenes in the cytoplasm and nucleus. *Science* **230**(4727), 770–776.

Weinberg, R. A. (1989). Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res.* **49**(14), 3713–3721.

Whitby, D., Howard, M. R., Tenant-Flowers, M., Brink, N. S., Copas, A., Boshoff, C., Hatzioannou, T., Suggett, F. E., Aldam, D. M., Denton, A. S., *et al.* (1995). Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet* **346**(8978), 799–802.

Wilkinson, D., Sheldon, J., Gilks, C. F., and Schulz, T. F. (1999). Prevalence of infection with human herpesvirus 8/Kaposi's sarcoma herpesvirus in rural South Africa. *S. Afr. Med. J.* **89**(5), 554–557.

Williams, A. B., Darragh, T. M., Vranizan, K., Ochia, C., Moss, A. R., and Palefsky, J. M. (1994). Anal and cervical human papillomavirus infection and risk of anal and cervical epithelial abnormalities in human immunodeficiency virus-infected women. *Obstet. Gynecol.* **83**(2), 205–211.

Williamson, A. L., Brink, N. S., Dehaeck, C. M., Ovens, S., Soeters, R., and Rybicki, E. P. (1994). Typing of human papillomaviruses in cervical carcinoma biopsies from Cape Town. *J. Med. Virol.* **43**(3), 231–237.

Williamson, A. L., Marais, D., Passmore, J. A., and Rybicki, E. (2002). Human papillomavirus (HPV) infection in Southern Africa: Prevalence, immunity, and vaccine prospects. *IUBMB Life* **53**(4–5), 253–258.

Wood, C., and Harrington, W., Jr. (2005). AIDS and associated malignancies. *Cell Res.* **15**(11–12), 947–952.

Xi, L. F., Critchlow, C. W., Wheeler, C. M., Koutsky, L. A., Galloway, D. A., Kuypers, J., Hughes, J. P., Hawes, S. E., Surawicz, C., Goldbaum, G., Holmes, K. K., and Kiviat, N. B. (1998). Risk of anal carcinoma in situ in relation to human papillomavirus type 16 variants. *Cancer Res.* **58**(17), 3839–3844.

Xi, L. F., Toure, P., Critchlow, C. W., Hawes, S. E., Dembele, B., Sow, P. S., and Kiviat, N. B. (2003). Prevalence of specific types of human papillomavirus and cervical squamous intraepithelial lesions in consecutive, previously unscreened, West-African women over 35 years of age. *Int. J. Cancer* **103**(6), 803–809.

Xu, D., Coleman, T., Zhang, J., Fagot, A., Kotalik, C., Zhao, L., Trevidi, P., Jones, C., and Zhang, L. (2007). Epstein-Barr virus inhibits Kaposis sarcoma-associated herpesvirus lytic replication in primary effusion lymphomas (submitted for publication).

Yang, T. Y., Chen, S. C., Leach, M. W., Manfra, D., Homey, B., Wiekowski, M., Sullivan, L., Jenh, C. H., Narula, S. K., Chensue, S. W., and Lira, S. A. (2000). Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma. *J. Exp. Med.* **191**(3), 445–454.

Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R., and Howley, P. M. (1985a). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am. J. Pathol.* **119**(3), 361–366.

Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R., and Howley, P. M. (1985b). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am. J. Pathol.* **119**(3), 361–366.

Yoshizaki, T., Sato, H., Furukawa, M., and Pagano, J. S. (1998). The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus latent membrane protein 1. *Proc. Natl. Acad. Sci. USA* **95**, 3621–3626.

Yoshpe, N. S. (1995). Oral and laryngeal papilloma: A pediatric manifestation of sexually transmitted disease? *Int. J. Pediatr. Otorhinolaryngol.* **31**(1), 77–83.

Zhang, L., and Pagano, J. S. (1999). Interferon regulatory factor 2 represses the Epstein-Barr virus BamH I Q latency promoter in type III latency. *Mol. Cell Biol.* **19**, 3216–3223.

Zhang, J., Das, S. C., Kotalik, C., Pattnaik, A. K., and Zhang, L. (2004a). The latent membrane protein 1 of Epstein-Barr virus establishes an antiviral state via induction of interferon-stimulated genes. *J. Biol. Chem.* **279**(44), 46335–46342.

Zhang, L., Hong, K., Zhang, J., and Pagano, J. (2004b). Multiple signal transducers and activators of transcription (STATs) are induced by EBV LMP-1. *Virology* **323**, 141–152.

Zhang, L., Zhang, J., Lambert, Q., Der, C. J., Del Valle, L., Miklossy, J., Khalili, K., Zhou, Y., and Pagano, J. S. (2004c). Interferon regulatory factor 7 is associated with Epstein-Barr virus-transformed central nervous system lymphoma and has oncogenic properties. *J. Virol.* **78**(23), 12987–12995.

Zimber-Strobl, U., and Strobl, L. J. (2001). EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes. *Semin. Cancer Biol.* **11**(6), 423–434.

Zippin, C., and Lum, D. (1993). Study of completeness of the surveillance, epidemiology and end results (SEER) program case ascertainment by hospital size and casefinding source. *Health Rep.* **5**(1), 87–90.

zur Hausen, H. (1999). Papillomaviruses in human cancers. *Proc. Assoc. Am. Physic.* **111**(6), 581–587.

zur Hausen, H. (2000). Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* **92**(9), 690–698.

zur Hausen, H., Gissmann, L., Steiner, W., Dippold, W., and Dreger, I. (1975). Human papilloma viruses and cancer. *Bibl. Haematol.* (43), 569–571.

zur Hausen, H., de Villiers, E. M., and Gissmann, L. (1981). Papillomavirus infections and human genital cancer. *Gynecol. Oncol.* **12**(2 Pt 2), S124–S128.

This page intentionally left blank

Index

Abacavir (ABC), 29, 131
  drug in HIV treatment, 270
  therapy, side effects of, 131
Acquired immunodeficiency syndrome (AIDS), 169, 509
  clinical trials, for HIV-1 vaccine, 435
  epidemiology of, 509, 533–535
  and estimation of patient, 229
Adeno-associated virus (AAV), 435
Adenosine deaminase deficiency (ADA), 307
Adenovirus-5, 435
AIDS-associated lymphomas, 520–524. *See also* AIDS-associated malignancies
  EBV role in, 520–521
    EBNA-1 and EBNA-2 protein, 523–524
    LMP 1 and 2, 520–524
AIDS-associated malignancies. *See also* AIDS-associated lymphomas; AIDS-associated NHL
  AIDS and, 512–513, 515
  carcinomas, 510
  Kaposi's sarcoma (KS)
  KSHV epidemiology, 515–517
  and oncogenesis, 516, 518–519
AIDS-associated NHL, 527. *See also* AIDS-associated malignancies
  PCNSL, 525
  PEL, 525–526
  systemic, 526–527
AIDS Clinical Trials Group (ACTG) protocol 076, 216, 460–462
AIDS prevention strategy
  microbicides
    characteristics, 233, 237–238
    development, 234
    intervention targets, 235
    role, 230
  retroviral zinc fingers of HIV-1 NCp7 role, 238–240
  SAMT chemotype characteristics, 240–244
  SAMT NCp7 inhibitors target, 246–249
  thioesters application, 244–246
  topical microbicides and prophylactic vaccine role, 232
AIDS-related lymphomas (ARL), 511
Amdoxovir, 133
Amino acid substitutions
  antiviral therapy and, 178
  in HIV-1, 174
  in noncleavage site mutants, 181
Amprenavir/Fosamprenavir
  adverse effects and drug interactions, 48
  pharmacokinetics, 47–48
Anal cancers, 510, 527–528
Antibody-mediated immunity, for HIV-1 vaccine, 436–441
  and antibody responses, 438–440
  neutralizing antibodies and, 440–441
Anti-CD4 antibody, 97–98
Antigen presenting cells (APCs), 434
Antigen-specific CD4 and CD8 T cells, markers, 80–81

Anti HIV-1
  activity
    AD101 resistance, 260
    BMS-802, 264
    entry inhibitors, 259–269
    integrase inhibitors, 276–278
    maraviroc-resistant virus, 262
    NNRTIs role, 274–276
    NRTIs role, 269–276
    protease inhibitors, 278–283
    RANTES derivatives, 263
    SCH-D-resistant clone and TAK-648 resistant, 261
    tenofovir drug and lamivudine therapy role, 273
  therapeutics, 129, 217
Antiretroviral therapy (ART), 453
  development of, 170
  drugs, 257–258, 266, 268, 275
  HIV-1 replicative cycle and, 172
  initiation of, 176
Antisense RNAs in HIV-1 infection prevention, 304
Antiviral compounds
  drugs, effects of, 172
  as topical microbicides
    efficacy of, 111
    PSC-RANTES, 110
Antiviral therapy, 78, 85–86
Aplaviroc
  binding and mechanism of action, 107
  E279 mutation, 107
  structure of, 101
Apoptosis signal-regulating kinase 1 (ASK1), 340
Apricitabine, 132
APV, drug, 184
AR-177 inhibitor in clinical trial, 216
Array technology, 328
Aspartic proteases, 172
Atazanavir
  adverse effects and drug interactions, 49
  pharmacokinetics, 48–49
3′-Azido-3′-deoxy-5′-O-β–D-glucopyranuronosylthymidine (GZDV), 29

Bacterial pneumonia, 485
Barrier-to-autointegration factor (BAF), 202
B-cell activation markers, CD23, CD30, CD40, CD71, 521
bcl-6 gene rearrangement, 520
BILR 351-BS, NNRTI, 145–146
BMS 378802 and BMS 488039 (small molecule inhibitors of gp120), 97
BMS-802 role in HIV treatment, 264
Body cavity-based lymphomas (BCBL), 514
Bovine papillomavirus (BPV), 532
Brecanavir drug role in HIV treatment, 280
Burkitt's lymphoma (BL) tumors, 521, 526

Caffeic acid phenethyl ester (CAPE), 211
(+)-Calanolide A, 145
Canarypox, 436
CCHC. *See* Cys-Xaa⊂2-Cys-Xaa⊂4-His-Xaa⊂4-Cys
CCR5 receptors, 371, 373, 385. *See also* HIV treatment, drugs in development
  aminooxypentane-RANTES (AOP-RANTES), 102
  CCR5 inhibitors, 63
  chemokine receptor, 98
  and CXCR4 resistance, 259–264
  humanized monoclonal antibodies, 102–103
  small molecule antagonists
    TAK 775 and SCH-C(or SCH351121), 103–106
$CD4^+$ and $CD8^+$ thymocyte, 401
CD4-binding pocket, 96
$CD4^+/CCR5^+$ positive T cells, 487
CD4 cell count, 15, 53, 56–57, 59
$CD8^+$ cells, 458, 486
$CD34^+$ cells, 354
$CD4C/HIV^{nef}$ Tg mice, 411
CD4C/HIVwt mouse, 410
CDK2/cyclin E, 335
cdk9 gene, 348
$CD4^+$ lymphocyte counts, 453, 455, 458
CD4-positive cells
$CD45RA^+$ $CD4^+$ T cells, 487
$CD45RA^+$ T cells, 488
CD4 receptors, 373, 385
$CD45RO^+$ T cells, 488
CD4 T-cell, 78–80, 82–86
  antigen exposure and functional profile, 84, 86
  gag and env-specific responses, 80
  T_{CM} and T_{EM} phenotypes of, 83
CD8 T-cell, 75–76, 78–87
  antigen exposure and, 86
  functional profile of virus-specific cells, 84

HIV-1-specific T_{ET}, 83
protective role of HIV-1-specific, 79
CD^+ T cells, 520
$CD3^+$ T cells, 405
$CD4^+$ T cells, 344, 370–371, 373, 382, 388, 407–411, 428–429, 431–434, 436, 441, 464–465, 485, 487
in blood and mucosal sites, 376
irreversible depletion of memory, 378–379
CD8 T cells, 427, 434
$CD8^+$ T cells, 378, 384, 408–409, 428–429, 436, 487
$CD4^+$ T lymphocyte (CTL), 301–307, 315, 371, 374
B-Cell lymphomas, 510, 515
Cell-mediated immunity, 75–76
B Cells, 405, 409
Cell surface markers, CD40, CD80, CD83, CD86, and CD25, 344
Cellular protein interactions
Gag, 340–343
Nef, 337–340
Tat, 332, 335–337
Cellulose acetate phthalate (CAP) virucidal activity, 236
Cervical cancers, 512, 527, 534
Cervical intraepithelial neoplasia (CIN), 534–535
cFos gene, 348
CGD. *See* Chronic granulomatous disease
C (HCV) viruses, 76
Chemokine receptors. *See* Coreceptors
Chimeric SIV-HIV (SHIV) viruses, 433
ChIP-chip analysis, 330
Chromatin immunoprecipitation (ChIP), 329
Chronic granulomatous disease, 307
Circulating Recombinant Form (CRF), 2, 6
Cleavage site mutants, 180–181
c-myc gene rearrangement, 520
Coinfection, 17
Col-1488 microbicides, 235–236
Combination chemotherapy (NRTIs) and monotherapy, 170
Combivir®, 29, 54
Concanavalin A (Con A) dose in HIV, 263
Conjunctival cancers, 527
Coreceptor binding targeting
CCR5 receptor
humanized monoclonal antibodies for, 102–103
peptide inhibitors for, 102
small molecule antagonists for, 103–106
CXCR4 receptor
peptide inhibitors for, 100
small molecule antagonists for, 100–102
Coreceptors
CCR5 and CXCR4, 98–99
CCR5 inhibition
peptide for, 102
small molecule for, 100–102
CXCR4 inhibition
antibody for, 102–103
peptide for, 102
small molecule CCR5 antagonists, 103–107
fusin, 98
Cryptosporidium enteritis, 485
CXCR4, chemokine receptor, 98
AMD070, CXCR4 antagonist, 101–102
peptide inhibitors of, 100
small molecule antagonists of, 100–101
CXCR4 coreceptor, 371
Cyanovirin N (CV-N), 263
and gp120 surface, 96
Cyc202, effects on viral gene expression, 330, 347–348
Cyclin A1, 345
Cyclin E/CDK2, 347
Cyclin T1, 335–336
Cyclin T/CDK9 complex, 335, 345, 347
Cyclophilin A (*CyPA*) gene, 336
CYP3A4, isoenzyme, 175
Cys-Xaa_2-Cys-Xaa_4-His-Xaa_4-Cys, 238
Cytochrome P446 system (CYP), 37
Cytomegalovirus (CMV) infections, 76, 374, 513
Cytotoxic T-lymphocytes (CTL), 374, 384, 407, 481–482, 486

Darunavir drug
activity against multi-PI-resistant mutations, 185
adverse effects and drug interactions, 51
HIV-1 resistance to, 186
pharmacokinetics, 51
role in HIV treatment, 280
Data Safety Monitoring Boards, 234
DDDP activity. *See* DNA-dependent DNA polymerase activity
ddI (2′, 3 ′-Dideoxynucleoside didanosine), 129–130

Delavirdine (DLV) drug
  adverse effects and drug interactions, 41
  HIV infection prevention role of, 275
  pharmacokinetics, 41
delta-32 CCR5 mutation, 99
Dendritic cells (DCs), 344, 407
Didanosine drug
  adverse effects, 34–35
  drug interactions, 35
  drug role in HIV treatment, 29, 270
  NRTI, 129–130
  pharmacokinetics, 34
Diketo acid (DKA), 211, 214
Discordant response, 282
2,2′-Dithiobis (benzamides) (DIBAs), 240
DKA derivative inhibitor in clinical trial, 216
DNA-dependent DNA polymerase activity, 123
DNA polymerase-γ, 28
Drug-resistant mutants, 60
Drug-resistant viruses determination, 275
DSMB. *See* Data Safety Monitoring Boards

EBV-encoded nonpolyadenylated nuclear RNAs 1 (EBER-1) and (EBER-2), 521
Efavirenz (EFV) drug
  adverse effects, 40
  drug interactions, 40–41
  pharmacokinetics, 39–40
  role in HIV infection prevention, 275
Elvucitabine drug, 133
Emtricitabine (ETC) drug
  adverse effects and drug interactions, 33
  NRTI, 131
  pharmacokinetics, 32–33
  role in HIV treatment, 270
Enfuvirtide (ENF) drug, 259, 264–269, 280
  fusion inhibitor, 108
  role in HIV-1 infection inhibition, 313
  role in HIV treatment, 264–266
env gene, 6, 329, 405, 411, 425, 433, 465, 467–468
  gp120 crystal structure, amino acid location, 260
  HIV-1 impact on fitness of virus, 267–268
  pseudotyped viruses role in ENF removal, 269
Enzyme-linked immunosorbent assay (ELISA), 4
Epstein-Barr Nuclear Antigen LP (EBNA-LP) types, 521, 524
Epstein-Barr virus (EBV), 76, 509, 512, 514–516, 520, 533
  infections, 485
  pathogenetic role in PCNSL, 525
Epzicom®, 55
Escrt1 gene, 336
Etravirine (TMC125), 144–145
Expanded access programs (EAP), 62

Fas-mediated apoptosis, 340
Fowlpox, 436
Funalenone IN inhibitor, 217
Fusion inhibitor
  for gp41, 107–108
  membrane fusion inhibition, 108–109
FZ-41, antiviral activity, 216

gag gene, 6, 329, 411, 425, 465–466
  and budding, 350–351
  HIV-1 infection inhibitor, 311
  p6 accessory protein, 343
  p24 capsid and p7 nucleocapsid, 342–343
  p17 matrix, 342
Gag-Pol precursors autocatalysis, 239
Gag protein, noncleavage and nonactive sites, 179–181
G190A mutation and NNRTI resistance, 148
Gastrointestinal tract cancers, 510
Gene expression microarrays, 332
Genetic disorder treatment by gene therapy, 307
Genital cancers, 527, 534
Genomics, 328
  biomarkers approach, 352–353
  cellular protein interactions of
    Gag, 340–343
    Nef, 337–340
    Tat, 332, 335–337
  cellular protein-protein interactions, 331–343
  drug target identification, 327–355
  Gag and
    p6 accessory protein, 343
    p24 capsid and p7 nucleocapsid, 342–343
    p17 matrix, 342
  Nef and
    apoptosis, 340
    CD4 downregulation, 338
    MHC-1 downregulation, 339–340

RNA interference approach, 353–354
Tat and
CBP/p296 interactions, 336–337
Cyclin T/CDK9 interactions, 335–336
therapies and, 328, 330–331
viral and induced cellular alterations, 343–351
Gag and budding, 350–351
Nef altering cell surface, 348–350
Tat effects on cellular transcription, 343–348
viral protein-protein interactions, 331–343
viral targets, 329–331
virion proteome approach, 351–352
GFAP-gp120 Tg mice, 412
GFAP promoter-driven HIV-1 *env* mice, 412
Glycoproteins, 78
gp41, N44* and C34 peptide inhibitor, 266
gp120 envelope (protein), 111
CD4-binding pocket, 96
coreceptor binding and conformational change, 107
fusion inhibitors for, 108
glycan shield formation, 95
Granzymes A and B, 428
GS-9313 and GSK-731, integrase inhibitor, 62
Guanosine analogue-abacavir
adverse effects, 33–34
drug interactions, 34
pharmacokinetics, 33
Guanosine quartet inhibitor in clinical trial, 216
Gums cancers, 528
Gut-associated lymphoid tissue (GALT), 429

Hairpin ribozyme role in HIV-1 infection prevention, 305
Heatshock protein 70 (HSP70), 344–345, 348
HEC. *See* Hydroxyethylcellulose
Hematopoietic cancers, 510
Hematopoietic progenitors role in HIV-1 infection prevention, 306
Hematopoietic stem cells (HSC), 401
Hepatitis B (HBV), 76
Herpes simplex virus, 76, 237
γ-Herpesvirus, 509, 515
Heteroduplex mobility assay (HMA), 11
Heterosexual transmission, 2
Hexim1, 335
HGS004 and HGS101, CCR5-specific humanized antibody, 103
High-grade B-cell NHL, 510
Highly active antiretroviral therapy (HAART), 298–299, 303, 306, 308, 315, 431, 461, 510–511, 525, 538
challenges in, 174
drawbacks in HIV treatment, 299
High mobility group chromosomal protein A1, 202
Histone acetyltransferases (HAT) p300, 337
HIV-1- associated nephropathy (HIVAN), 347
HIV-coreceptor interactions
CXCR4 inhibition, 98
ALX40-4C, 100
AMD070, antiviral potency of, 101–102
AMD3096, small molecule inhibitor, 99–100
HIV envelope glycoproteins
gp120 and gp41, 78
HIV genetic forms, methods for identification, 11
alternative methods, 11
phylogenetic sequence analysis, 11
HIV-1 IN
cellular integration, 202–210
inhibitor-binding site discovery, 214
inhibitors, 212–213
representative inhibitors, 214
retroviral integration chemistry, 201–202
structure, 200–201
HIV infection
chemokine receptors in
CCR5 and CXCR4 coreceptors, 98–99
fusin, 98
delta-32 mutation, impact of, 99
inhibition of
aminooxypentane-RANTES (AOP-RANTES), 102
CC chemokines for, 99
membrane fusion, 94
R5-tropic viruses, 99
viral entry process, 94
X4-tropic viruses, 99
HIV-1 infection, gene therapy in
drawbacks for, 314–316
early clinical trials, 300
antisense RNAs clinical trials, 304
dominant negative mutant of HIV Rev protein, 302–303
ribozymes clinical trials, 304–306

HIV-1 infection, gene therapy in (*continued*)
RRE sequences clinical trials, 303–304
HIV-1 internalization, 313–314
lentivirus vectors, 309–311
RNA interference, 311–313
HIV infections, distribution and estimated magnitude, 3
HIV-1 integrase, functional domains, 204
HIV integration inhibition
inhibitiors of HIV IN enzymatic activities, 210–216
PIC targeting, 216
HIV-1 prevention strategy
microbicides
characteristics, 233, 237–238
development, 234
intervention targets, 235
role, 230
retroviral zinc fingers of HIV-1 NCp7 role, 238–240
ribozymes role, 304–306
RRE role, 303–304
SAMT chemotype characteristics, 240–244
SAMT NCp7 inhibitors target, 246–249
tat/*rev* genes role, 304
thioesters application, 244–246
topical microbicides and prophylactic vaccine role, 232
HIV-1 resistance to NNRTIs
K103N and P221H mutation, 148
mutations associated with in residues 98–108, 147, 179–190
HIV-1 resistance to NRTIs
biochemical mechanisms of
selective alterations in NRTI binding, 134
K65R mutation, 137–138
L74V mutation, 138
M184V mutation, 136–137
NRTI cross-resistance
K65R mutation, 140
TAMs and HIV DNA synthesis, 139
Q151 mutation, 139
substitutions at position 69 in RT, 138
thymidine analog mutations, 134–136
V75T mutation, 138
HIV RNH inhibitors
BBNH and CPHM, 151
DHBNH, 152
HIV-1 RT
inhibitors of
HIV RT RNH, 150–151
obligatory heterodimer, 124
p51 subunit and p66 subunit of, 124–125
HIV-1 RT DNA polymerase activity
RT mechanistic forms and, 126
HIV-2 infection, 370, 424, 455
HIVs and genesis of HIV-1 pandemic, origin
HIV/AIDS, zoonosis, 13–14
origin of pandemic HIV-1 strains, dating, 14
HIV treatment
CCR5 antagonists for, 106
drugs in development, 61–63
entry inhibitors role in
CCR5 and CXCR4 resistance, 259–264
fusion inhibitors resistance, 264–266
virus resistant fitness, 268–269
FDA approval drugs
ENF, 264
NRTIs, 270
protease inhibitors, 278
tipranavir and darunavir, 280
fusion inhibitor for, 108
initial regimen
initial PI, 56–57
NNRTI vs PI, 55–56
NRTI choice, 54–55
PI monotherapy, 58–59
PI plus NNRTI (NRTI sparing), 59
three class regimens, 57–58
triple NRTI therapy, 58
initiation of therapy, 53–54
long-term management, 59–60
nonnucleoside reverse transcriptase inhibitors, 36–41
nucleoside/nucleotide reverse transcriptase inhibitors, 28–36
protease inhibitors, 41–52
resistance, 60–61
resistance pattern of entry inhibitors
CCR5 and CXCR4 antagonists, 109–110
fusion inhibitor, 109
tropical microbicides for, 110
HIV-1 vaccine
clinical trials and, 435
development strategies
antibody-mediated immunity, 436–441
for eliciting effective antibody responses, 438–440
live-attenuated HIV, 429–436
mimotope strategy to induce neutralizing antibodies, 440–441
T-cell-inducing vaccine, 434–435

neutralizing monoclonal antibodies, 438
requirements for, 428–429
HIV-1 variability and recombination
dual infection, superinfection, and recombination, 17–18
HIV-1 vertical transmission, 478–481
characterization, 462–464
chemokine receptors, 464–465
diagnosis in neonates and infants, 460
factors associated with, 458–460
functional domains of genes, 470–478
immunologically relevant mutations in isolates, 481–484
in infants, 453–485
molecular properties of HIV-1 from mother-infant pairs, 465–469
prevention, 460–462
timing and mechanism of, 456–458
HMGA1. *See* High mobility group chromosomal protein A1
Hodgkin's lymphomas, 509–510, 520–521
HPV-induced cervical cancers, 510
HSCs. *See* Human hematopoietic stem cells
HSV. *See* Herpes simplex virus
hu-CD4/CCR5 Tg rat, 409–410
Human hematopoietic stem cells, 303
Human herpesvirus 6A and 6B, 330
Human herpesvirus-8 (HHV-8), 509, 512, 514, 533
Human immunodeficiency viruses (HIV), 1. *See also* HIV-2 infection; Human immunodeficiency viruses (HIV-1)
emergence of HIV-1 recombinants, 11
genotypes and origins, 5
global HIV-1 variability, 9
gp41 transmembrane glycoprotein, 94
HIV-2, genotype classification and geographic distribution, 7
HIV-1s, genotype classification of, 4–5
recombinants, 6–7
subtypes, 6
HIV-1 variants
in Asia, 9–10
of geographical relevance, 10
infection
and CXCR4 variants, 371
epidemiology of, 509, 533–535
induced AIDS pathogenesis, 373–377, 381
life cycle, integration, 201
medications, 330–331
replication, NCp7 function, 239
transmission, 231
types (HIV-1 and HIV-2), 4
virion, 129
Human immunodeficiency viruses 1 (HIV-1), 258, 297, 370–371, 374, 510
amdoxovir, potent activity of, 133
amino acid substitutions in, 176
antiviral therapies, 327
cellular processes by alteration and subversion, 336
disease progression in infants, 485–490
distinct groups, 4
D67N and K70R mutations, 135
gene expression in T lymphocytes, 489–490
gene therapy, 298–300
genetic variability, 468–469, 478–479
gene transfer and hemapoietic progenitors, 306–309
genome
identification of cofactors in, 332
information in, 122
reverse transcription of, 331
group M viruses, 6
heterogeneity in, 468
hu-PBL-SCID mouse model, 401, 405–407
infection, 399–413, 427
gene therapy strategies, 300
immune response during, 427–428
natural history, 424–427
suppression steps, 300–302
transmission, 428–429
infection pathogenesis, Balb/C Rag$^{-/-}$ mice, model, 408
infection reduction, SAMT compound role, 240–241
isolates from nontransmitting mothers
molecular analyses of, 478–481
isolates from vertical transmission
immunologically relevant mutations in, 481–485
K65R mutation, 137
L74V mutation, 138
IN mutants, catalytic activities, 202–209
NCp7, retroviral zinc fingers, 238–240
nonprimate models, 399–413
Balb/C Rag$^{-/-}$ mice, 408
HIV-1 Tg mice, 410–412
humanized immune competent mice and rats, 409–410
hu-PBL-SCID mouse, 405–407
NOD/LtSz-SCID mice, 407–408

Human immunodeficiency viruses 1 (HIV-1), (*continued*)
NOD-SCID $\gamma C^{-/-}$ mice, 408
rabbit model, 412–413
$Rag2^{-/-}$ $\gamma C^{-/-}$ mice, 408
SCID-hu Thy/Liv mice, 401–405
ORFs, 467
pathogenesis, 485–490
prevention strategy
antisense RNAs role, 304
gene therapy strategies, 300
HSCs role, 303
lentivirus role, 309–311
protease
active site mutants of, 179
active site of, 171–172
aspartyl protease, 170
β-hairpin region, 171
cleavage site sequences of, 172
HIV Gag and Gag-Pol polyproteins, cleavage of, 170–171
substrate binding and PI binding to, 184
Val82Ala mutation, 179
protease, active site mutants, 179
protease, β-Hairpin region, 171
proviral genome, 425
Q151 mutation, 139
recombinants
circulating recombinant form, 6
unique recombinant form, 6–7
replication cycle, 238
replication in T lymphocytes, 488–489
resistance to
AZT, 135
darunavir, 186–188
NRTIs, 133
3TC, 130
RT dimerization inhibitors, 153
RT interaction with NNRTIs, 274
RT reduction, 273
subtypes, rates of progression to AIDS, 15
Tg mice model, of HIV 1 infection pathogenesis, 401, 410–412
treatment
antiretroviral therapy, 258
BMS-802 role, 264
entry inhibitors, 259–269
integrase inhibitors, 276–278
lamivudine drug, 270
NNRTIs role, 274–276
NRTIs role, 269–276
protease inhibitors, 278–283
Rev protein role, 302–303
tenofovir drug and lamivudine therapy role, 273
variability and recombination, biological implications of recombination, 17
subtypes and disease progression, 14–16
viral genes, 329
viral proteins and their cellular-interacting proteins, 332–333
V75T mutation, 138
Y115F mutation, 139
Humanized immune competent mice and rats model, of HIV 1 infection pathogenesis, 409–410
Human papilloma virus (HPV), 76, 509
associated cancers, 527–529
capsid and vaccine, 532–533
causative agent, 529–538
epidemiology of, 533–535
genome structure, 529–530
induced transformation and cancer progression, 535–538
life cycle, 530, 532
Human renin inhibitors, 172
Human T-cell leukemia virus, 330
Human T-lymphotropic virus 1 (HTLV-1), 434
Humoral immunity, 75–76
Hydroxyethylcellulose, 245

IgG1-b12 antibody, 407
Immune responses
HIV-1-specific T-cell responses, 78–87
CD4 and CD8 T-cell, phenotypic and functional profiles, 80
function, 83–86
kinetics in primary infection, 79–80
phenotype, 80–83
protective role, 78–79
specificity and breadth of, 86–87
humoral HIV-1-specific immune response, 77–78
Immunofluorescence antigen (IFA), 515
Immunoreceptor tyrosine-based activation motif (ITAM), 376, 516
IN-catalyzed reaction in HIV integration, 210–211
Indinavir drug
adverse effects, 45–46
drug interactions, 46
pharmacokinetics, 45

IN-DNA binding assays in HIV integration, 211
Infants
diagnosis of HIV-1 infection in, 460
HIV-1 pathogenesis and disease progression in, 485–490
HIV-1 vertical transmission in, 453–485
Infectious virus determination, 231
Injecting drug users (IDUs), 10
IN protein
C-terminal domain, 201
domains, 201
inhibitor discovery, 217
Integrase inhibitors role in HIV treatment, 62, 199–204, 210–212, 214–218
resistance, 278–279
viral fitness, 278–279
Interfacial inhibition, 211
Interferon-γ, 428, 486
Interferon receptors, 408, 428, 486
Interleukin receptor (IL), 408
Intracellular immunization, 309
Intrinsic resistance, 263
Invasive cervical carcinoma, 510
Investigational NRTIs
apricitabine, 132
elvucitabine and amdoxovir, 133

Janus kinase 3 (JAK3), 522
JR-CSF Tg mice, 411
c-JunN-terminal kinase, 522

Kaposin gene, 516–518
Kaposi's sarcoma (KS), 509, 516–519, 534
AIDS-associated, 512–513, 515
associated herpesvirus (*see* KS-associated herpesvirus (KSHV))
endemic, 512–513
HIV-associated, 512–513
iatrogenic, 512–513
KSHV epidemiology and, 515–516
viral etiology in, 513–515
viral oncogenesis, 516, 518–519
KLEAN study, 56. *See also* HIV treatment, initial regimen
K103N and P221H mutation, 147–148
K65R mutation, 137–138
KS-associated herpesvirus (KSHV), 330, 509, 512, 514, 518, 526, 533
epidemiology, 515–517

Lamivudine (3TC) drug, 29
adverse effects and drug interactions, 32
NRTI, 130
pharmacokinetics, 32
role in HIV treatment, 270
therapy role in HIV-1 treatment, 273
Larynx cancers, 528
Latency-associated nuclear antigen (LANA), 516–517, 519, 526
Latent membrane protein 2A (LMP-2A) and 2B (LMP-2B), 523
Latent membrane protein 1 (LMP-1) and (LMP-2), 521–522, 526–527
LEDGF. *See* Lens epithelium-derived growth factor
LEDGF/p75 targei in PIC, 215
Leiomyosarcoma, 512
Lens epithelium-derived growth factor, 202
Lentivirus vectors role in HIV-1 infection prevention, 309–311, 329
L100I mutation, 147
L-866,806 inhibitor in clinical trial, 216
Live-attenuated HIV, for HIV-1 vaccine, 429–436
Long terminal repeats, 15, 200, 202, 304
Long-term nonprogressors (LTNPs), 77, 352–353
Lopinavir drug
adverse effects and drug interactions, 47
pharmacokinetics, 47
LPV/r, 55–57, 59. *See also* HIV treatment, initial regimen
Lung cancers, 510, 528
L74V mutation, 138
Lymphoblastoid cell lines (LCLs), 521
Lymphocrytovirus, 515
Lytic gene (K-RTA), 526

Major gene therapy clinical trials, 301
Major histocompatibility complex (MHC), 339
Major histocompatibility complex class I (MHC-1), 383–384
M184 amino acid role in HIV-1 RT, 271–272
Maraviroc-resistant virus, 262
Maximum tolerated dosage, 241
MDR protease and PI binding, 183–184
Measles, 429
Membrane fusion inhibition
SP-01A, small molecule entry inhibitor, 108–109
between viral envelope and cell, 108

Membrane proximal external region (MPER), 78
Memory T cells, 80
MGMT. *See* O (6)-methylguanine-DNA-methyltransferase
MHC-II+ cells, 401
Microbicides
  characteristics, 233, 237–238
  developmental strategy, 234, 237
  intervention targets, 235
  as membrane disruptors, 110
  role in AIDS prevention, 230–231
  topical, 230–238, 241, 244–246
  virucides, 236
microRNA (miRNA), 312
Mimotope strategy and antibodies, 440–441
Minimum inhibitory concentration (MIC), 37
MIP-1α and 1β, 428, 519
Mitochondrial DNA (mtDNA), 28
Mitogen-activated protein kinase (MAPK), 518
MK-514, integrase inhibitor, 62–63
MLV. *See* Murine leukemia virus
M87o inhibit HIV-1 infection, 313
Molecular epidemiology, 2
Molecular evolutionary genetic analysis (MEGA), 11
Monotherapy, 133
Mouse-human heterokaryon formation, 409
Mucocutaneous herpes simplex virus infection, 485
Mucosal and esophageal candidiasis, 485
Multicentric angiofollicular lymphoid hyperplasia, 514
Multicentric Castleman's disease (MCD), 514
Multidrug-resistant, 243
Multidrug-resistant mutations, 272
Multinucleated giant cells (MNGCs), 379, 382
  histopathology and SIV-specific *in situ* hybridization, 381
Multiple drug combination therapies, 139
Mumps, 429
166-Murine HIV-1 transgenic spleen cells, virus measurement, 243
Murine leukemia virus, 310
M184V mutation, 63. *See also* HIV treatment, drugs in development
M184V mutation and 3TC monotherapy, 136–137
M184V viral mutation, 258
Mycobacterium avium infection, 374, 485
Mycobacterium tuberculosis, 411
Myelosarcomas, 510

Naphthyridine carboxamide inhibitor in clinical trial, 215
Nasopharyngeal carcinoma (NPC), 510, 521
Natural products as IN inhibitors, 216–217
Nef-binding protein-1 (NBP1) protein, 336, 338
nef gene, 330, 384, 410, 411, 425, 431–432, 435, 454, 465–470, 475, 477–478, 484
  altering cell surface, 348–350
  apoptosis and, 340
  CD4 downregulation and, 338
  MHC-1 downregulation and, 339–340
Nelfinavir drug
  adverse effects and drug interactions, 46–47
  pharmacokinetics, 46
  role in HIV treatement, 281
Neuro-AIDS and SIVE, 374
Nevirapine (NNRTI) drug, 142–143
  drug interactions, 39
  pharmacokinetics and adverse effects, 38
  role in HIV infection prevention, 275
NFκB gene, 348
NFL protein, 353
NK cells, 405, 406, 427
NL4-3 Δ*gag-pol* Tg mice, 410
NNRTI and NRTI resistance mutations, interactions between, 148–149
NNRTI-binding pocket (NNRTI-bp), 275
NNRTIs inhibit HIV-1 RT binding, 274–276
NOD/LtSz-SCID mice model, of HIV 1 infection pathogenesis, 407–408
NOD-SCID $\beta 2^{mnull}$ mice, 408
NOD-SCID $\gamma C^{-/-}$ mice model, of HIV 1 infection pathogenesis, 408, 413
Nonactive site mutations, 178
Noncleavage site mutants, 181
Non-Hodgkin's lymphomas (NHL), 510–512, 520, 534
Non-nucleoside inhibitor binding pocket
  NNRTI binding, 140
    conformational changes in, 141
    primer grip, structural elements, 141
    thumb subdomain mobility, 142
    T/P translocation, 141–142
  RT residues, side chains of, 140

Non-nucleoside reverse transcriptase inhibitor (NNRTI), 236, 461
adverse effects, 37
binding and/or incorporation, selective alterations in, 134
for clinical use
delavirdine (DLV), 143
efavirenz (EFV), 143–144
nevirapine (NVP), 142–143
cross-resistance
K65R mutation, 140
TAMs and HIV DNA synthesis, 139
delavirdine, 41
drug interactions, 37–38
drug resistance (*see* HIV-1 resistance to NRTIs)
efavirenz, 39–41
HIV-1 resistance to
K103N and P221H mutation, 148
mutations in residues 98–108, 147, 179–190
investigational
BILR 351-BS, 145–146
(+)-Calanolide A, 145
etravirine(TMC125), 144–145
mechanism, 36–37
mechanisms, HIV resistance, 270–271
mechanisms of inhibition
dNTP incorporation, 141
interaction with NNIBP, 141
T/P translocation, 141–142
as microbicides
HIV transmission inhibition, 149
UC777, anti-HIV microbicide, 150
nevirapine, 38–39
resistance, 274–275
RT allosteric inhibitors, 122, 140
viral fitness, 275–276
Nonpeptidic PIs (NPPIs), chemical moiety, 188
Nucleocapsid protein (NCp7), 230
structural elements, 238
zinc finger domains, computational analysis, 248
Nucleocapsid protein (NCp7) zinc fingers interaction and NMR experiments, 248
Nucleoside analog prodrug, 133
Nucleoside/Nucleotide reverse transcriptase inhibitors
adenosine analogues, 34–36
adverse effects, 28–29
cytosine analogues
emtricitabine, 32–33
lamivudine, 32
guanosine analogue-abacavir, 33–34
mechanism of action, 28
resistance, 269–272
thymidine analogues
stavudine, 31–32
zidovudine, 29–31
viral fitness, 272–274
Nucleoside reverse transcriptase inhibitor (NRTI), 460
as anti-HIV therapeutics, 129
for clinical use
abacavir (ABC) and emtricitabine, 131
retrovir (AZT), 129
tenofovir (Viread), 131
videx, 129–130
zalcitabine, stavudine, and 3TC, 130
exonucleolytic "proofreading" activity, 128–129
HIV-1 infection treatment, 127–128
HIV-1 resistance to
biochemical mechanisms of, 134
drug-resistant mutants of HIV-1, 133
K65R mutation, 137–138, 140
L74V mutation, 138
M184V mutation, 136–137
NRTI cross-resistance, 139–140
Q151M complex, 139
selective alterations in NRTI binding, 134
substitutions at position 69 in RT, 138
thymidine analog mutations, 134–136
V75T mutation, 138
investigational
apricitabine, 132
elvucitabine and amdoxovir, 133
mechanisms of NRTI inhibition
chain-termination, 129
phosphorylation of, 127
RT-catalyzed DNA synthesis inhibition, 127–128
structures of, 128

Oligonucleotide microarray, 328
O (6)-methylguanine-DNA-methyltransferase, 315
Opportunistic infections (OIs), 374
Oral cancers, 510, 527
Otitis media, 485

PAK1, PAK2 and PAK3 kinases, 354
Palate cancers, 528
Pantroglodytes troglodytes, 13
Papillomavirus genome structure, 529–531
PARP. *See* Poly(ADP-ribose) polymerase-1
PATEs. *See* Pyridinioalkanoyl thioesters
PBLs. *See* Peripheral blood lymphocytes
PBMCs. *See* Peripheral blood mononuclear cells
Penicillium sp., 218
Penile cancers, 510, 528, 542
Peptide inhibitors. *See* Peptidic inhibitors
Peptidomimetics, 188
Perforin, 428
Peripheral blood lymphocytes, 262
Peripheral blood mononuclear cell, 261, 303, 306, 316
Pharmacological inhibitor, clinical trials, 215–216
Phenotypic markers, relationship, 81. *See also* Immune responses
Phosphatidylinositol 3-kinase (PI3-K), 523
Phosphofurin acidic cluster sorting protein (PACS1), 336, 340
Phosphorylated positive transcription elongation factor b (P-TEFb), 335–336
Pluripotent microbicide role, 237
p53 mutations, 520
Pneumocystis carinii pneumonia, 485
pol gene, 329, 411, 425, 454, 465, 467–470
Poly(ADP-ribose) polymerase-1, 247
Polyclonal B-cell lymphoproliferative disorder, 514
Polymorphism, 178
Postgenomic research techniques, 332
Poxviruses, 330
p66-p51 subunit association, 153
Preclinical development inhibitors, 216–217
Preclinical microbicide development, 235
Preintegration complex (PIC), 201–202, 204, 206, 208–211, 214–215, 331
  function and co-factors, 210
  target in HIV integration inhibition, 215
Primary central nervous system lymphoma (PCNSL), 509–511, 524–525
  EBV pathogenetic role in, 524
Primary effusion lymphoma (PEL), 514, 519, 524–526
Pro-140, CCR5-specific humanized monoclonal antibodies, 102
3′-P role in ST and HIV integration, 211, 215
Prophylactic HPV vaccine, 510
Prophylactic vaccine role in HIV reduction, 232
Pro-538 (recombinant fusion protein)
  broad spectrum neutralization activity against HIV, 96
Protease inhibitors (PIs)
  activity against drug-resistant HIV-1
    primary mutations and cross-resistance, 182
    Val82Ala mutation and PI binding, 183
  amprenavir/fosamprenavir, 47–48
  atazanavir, 48–49
  boosting of pharmacokinetics parameters of
    clinical efficacy and, 175–176
    RTV and CYP3A4, 175
  CYP3A4 system and, 175
  darunavir, 51
  drug resistance
    active site mutations, 177
    specificity-determining residues, mutations of, 177–178
  fusion inhibitor-enfuvirtide (T-20), 51–52
  HIV-1 PIs, pharmacokinetic limitations of, 174
  indinavir, 45–46
  lopinavir, 47
  mechanism action , 41–43
    posttranslational processing, 170
    substrate-binding cleft, 171
  nelfinavir, 46–47
  primary mutations
    Asp25Asn mutation, 183
    PI binding, 179
    Val82Ala mutation, 182–183
  ritonavir, 43–44
  role in HIV treatment resistance, 278–281
    viral fitness, 281–283
  saquinavir, 44–45
  symmetry-based, 172, 174
  tipranavir, 49–50
  TMC-126 and darunavir, 185
  viral resistance to
    active site mutations, 177
    amino acid substitutions, 178
    specificity-determining residues mutations, 177–178
Protein phosphatase 2A (PP2A) inhibitor, 344–345
Proteomics, drug target identification, 327–355

Provirus, 200
p51 subunit
  molecular crowbars and, 153
  protein interaction with p66 subunit, 125
  residue E138 of, 148
p66 subunit
  interactions between, 125
  mechanisms of NNRTI inhibition, 140–142
  molecular crowbars and, 153
  polymerase active sites of, 125
  structure of, 124
Pyridinioalkanoyl thioesters, 240

Q151 mutation and NRTI resistance, 139
Q151M mutation in HIV-1 RT, 272

Rabbit model, of HIV pathogenesis, 412–413
Rac/Rho-GDI complex, 344
Rag2$^{-/-}$ $\gamma C^{-/-}$ mice model, of HIV 1 infection pathogenesis, 401, 408, 413
RANTES, 428, 519
Rapid progressor (RP) macaques
  SIV evolution in, 385–387
    biological properties of virus, 385, 387
    molecular changes, 385–386
  SIV virus role in, 387–388
  survival rates for SIV-infected, 374–375
  transient immune responses, 378
ras gene mutations, 520
Rattus norvegicus, 409
RBP-Jk protein, 524, 526
Recombination Identification Program (RIP), 12
Replication competent lentivirus (RCL), 309
Rev-binding protein 2, 345
Reverse transcriptase, 121
  conformational change of, 126
  DNA polymerase activity of, 123
  polymerase active sites and catalytic aspartate residues, 125
  RT–DNA T/P binary complex and RT–DNA T/P–dNTP ternary complex, 126–127
Reverse transcription initiation inhibitors, 153–154
rev gene, 330, 425, 435, 454, 466–470, 474–475
RevM10 advantages and disadvantages, 303
Rev protein role in HIV-1 treatment, 302
Rev-response element, 300–304
Rhadinovirus, 515
Ribozymes role in HIV-1 infection prevention, 304–306
Ritonavir
  adverse effects and drug interactions, 44
  pharmacokinetics, 43–44
Ritonavir and saquinavir based therapy for HIV, 282
RNA-dependent DNA polymerase activity, RT, 123
RNA interference (RNAi), 298
  approach, in genomics, 353–354
RNAi role in HIV-1 infection role, 311–313
RNA Pol II CTD, 335, 347
RNA polymerase II (RNA Pol II), 332, 335–336
RRE. *See* Rev-response element
RRE role in of HIV-1 infection prevention, 303–304
RT-catalyzed DNA synthesis inhibition, 127–128
RT-directed mutagenic inducers, 154
RT DNA polymerase activity sites, 125
RT-DNA T/P binary complex, 126–127
RT-DNA T/P-dNTP ternary complex, 126–127, 135
RT inhibitors
  nonnucleoside HIV inhibitors (NNRTIs), 122
  nucleoside RT inhibitors (NRTIs), 122
RTI-resistance mutation
  and effect on viral fitness, 271–272
  in HIV-1 RT crystal structure, 271
RT ribonuclease H (RNH) activity inhibitors, 123–124
R5-tropic viruses, HIV infection, 99
RTV and SQV (PIs), 175
Rz2 hammerhead ribozyme role in HIV-1 infection prevention, 305

S-Acyl-2-mercaptobenzamides (SAMTs), 239–250
  antiviral activity to HIV-1, 246
  chemotype characteristics, 240–244
  interaction with NCp7 zinc finger, 248–249
  NCp7 inhibitors target, 246–249
  synthesis, 244
  thioester compounds mechanism, 247
SAMT-89 and 243 assessment in virus transmission inhibition, 243–245

SAMT-19 dose in HIV treatment, 241–242
Saquinavir
  adverse effects and drug interactions, 45
  pharmacokinetics, 44–45
sCD4 protein
  binding with gp120, 96
scFV. *See* Single-chain variable fragments
SCH-C (or SCH351121), small molecule CCR5 antagonist
  oral bioavailability of, 103
  putative binding site of, 106
  transmembrane domains, mutation of, 107
SCH-D-resistant clone, 261
SCID-hu mice, 406
SCID-hu Thy/Liv mice model, of HIV 1 infection pathogenesis, 401–405, 411
SDF-1. *See* Stromal cell-derived factor 1
Secondary mutation, PI, 178–179, 182
Severe combined immunodeficiency (SCID), 436, 526
Sexually transmitted diseases (STDs), 16, 231, 533–534, 537–538
SFFV. *See* Spleen focus-forming virus
SHIV. *See* Simian/human immunodeficiency virus
Signal transducer and activator of transcription 1 (STAT-1) and (STAT-2), 521
Simian/human immunodeficiency virus, 237
Simian immunodeficiency viruses (SIVs), 7, 241, 400, 424, 456
Sinusitis, 485
SIV encephalitis (SIVE), 370–372, 383
SIV from sooty mangabey monkeys (SIV-sm), 400
  AIDS progression in, 374
  biology, 371, 373
  evolution in RPs, 385–387
    biological properties of virus, 385, 387
    molecular changes, 385–386
  genes involved in pathogenesis, 384–385
  and HIV-1, 371, 373
  host immune responses and MHC, 383–384
  pathogenesis
    immune activation variation, 377
    infection models for AIDS, 373–374
  rapid disease progression to AIDS due to, 369–388
  SIV-smPBj Nef protein, 376
  SIV-smPBj6.6 strain, 372
  SIV-smPBj14 strain, 372
  SIV-smPBj virus, 375–376
  SIV-smPG/mIn strain, 372, 381–382, 387
  strain genetic and pathogenic diversity, 371–373
  virus role in rapid disease, 387–388
SIV infection of macaques (SIV-mac)
  disease progression, 383
  host immune responses and MHC, 383–384
  immunologic features, 378–379
    effects on immune activation, 378–379
    irreversible depletion of memory $CD4^+$ T cells, 378
    transient immune responses, 378
  pathogenesis, 373–377
    acutely lethal SIV-smPBj, 375–376
    $CD4^+$ T cells in blood and mucosal sites, 376
    correlates of disease progression, 376–377
    disease progression, 374–375
    genes involved in, 384–385
    immune activation variation, 379
    infection models for AIDS, 373–374
    plasma viral load, 377
  pathological features, 379, 381–383
  SIV-mac235, 433–434
  SIV-mac247, 432, 434
  SIV-macC8, 431
  strain genetic and pathogenic diversity, 371–373
  strains, 370–372, 381, 387
  virological features, 379–380
SIVs-infected nonhuman primates, 13
Small molecule antagonists
  of CCR5
    TAK 775 and SCH-C, 103
  CMPD 167, 106, 110
  of CXCR4 receptor
    AMD070, 101–102
    AMD3096, 100
  mechanism of action , 107
Small molecule inhibitors. *See* Small molecule antagonists
Smallpox, 429
SMART trial, 59. *See also* HIV treatment, long-term management
SP-01A, small molecule entry inhibitor
  lipid rafts, disruption of, 109
  membrane fusion inhibition, 108
Spleen focus-forming virus, 310
SQL. *See* Styrylquinoline

Squamous cell neoplasm, 512
Staphylococcus, 411
Stavudine (d4T) drug, 29
  adverse effects, 31–32
  drug interactions, 32–33
  pharmacokinetics, 31
  role in HIV treatment, 270
Step-by-step pathway of virus in antiretroviral treatment, 280–281
STI and retroviral integration, biochemical steps, 203
ST inhibition determine, 210
Strand transfer inhibitors, 203, 211
Stromal cell-derived factor 1, 100
Structure-based PIs, nelfinavir (NFV) and amprenavir (APV), 174
Structured treatment interruption, 310
Styrylquinoline role in HIV IN inhibition, 215
Substrate-based inhibitors, 172
Sulfated polyanions, HIV prevention, 110
Symmetry-based inhibitors, design of, 172, 174
Syncytium-inducing (SI) isolates, 402
Synthetic IN inhibitors, discovery, 217
Systemic AIDS-associated NHL, 526–527

T-20. *See* Fusion inhibitor-enfuvirtide
TAK 775
  binding site of, 106
TAK-648 resistant in HIV-1, 261
TAMs. *See* Thymidine analogue mutations
Tat
  CBP/p296 interactions and, 336–337
  Cyclin T/CDK9 interactions and, 335–336
  effects on cellular transcription, 343–348
Tat/CBP/p296 transcription, 332
Tat/cyclin T/CDK9 transcription, 332
tat gene, 330, 425, 435, 454, 466–470, 473–474, 484
tat/rev genes role in HIV-1 infection prevention, 304
Tau protein, 353
T cell receptor (TCR), 400
T cells, 373
  inducing vaccine, 434–435
  lymphomas, 510
TCR Vβ repertoire, 401
TDM. *See* Therapeutic drug monitoring
T20 drug role in HIV-1 infection inhibition, 313
Tenofovir disoprovil fumarate drug
  adverse effects, 35–36
  drug interactions, 36
  pharmacokinetics, 35
  role in HIV treatment, 270, 273
Tenofovir, NRTI, 132
TFIID/TFIIA complex, 332
Tg $CD4^+$ T cells, 411
Therapeutic drug monitoring, 37
Thioester as microbicides, 244–246
Thymidine analogue mutations, 270
  D67N and K70R mutations, 135
  TAM mutation, 136
Tipranavir drug, 188
  adverse effects and drug interactions, 50
  antiviral activity of, 189
  binding motif and structure of, 189
  pharmacokinetics, 49–50
  role in HIV treatment, 280
T-lymphocyte ontogeny, 338
TMC-126, drug
  activity against protease resistance mutations, 186
  hydrogen bond interactions with protease backbone, 186
  pharmacokinetic properties of, 187
TMC-125 (etravirine), 62
TNF-α-mediated apoptosis, 340
TNFR-associated factors (TRAFs), 522
TNX-351, anti-CD4 antibody, 98
Tongue cancers, 528
Tonsils cancers, 527
Topical microbicides role in HIV reduction, 232
T/P translocation, 141–142
TPV. *See* Tipranavir
Trizivir®, 29
Tumor necrosis factor-α (TNF-α), 15, 340, 410
Tumor necrosis factor receptor (TNFR), 522

UC-777 virucidal activity, 236
Unique recombinant forms (URFs), 7
United Nations Program on HIV/AIDS (UNAIDS), 2
Upper respiratory tract cancers, 528

Vaccinia virus Ankara, 434
Vagina cancers, 528
Vaginal microbicides
  for HIV transmission inhibition, 149
  UC777 and HIV-1 infectivity, 150

Val82Ala mutation and PI binding., 183
V106A mutation, 147
Varicella-zoster virus, 330
Varicella zoster virus (VSV), 76
Vascular endothelial cell growth factors (VEGF), 519
Vicriviroc, 63. *See also* HIV treatment, drugs in development
vif gene, 330, 334, 425, 454, 465–470, 475, 480–481, 484
Viral and induced cellular alterations
  Gag using cellular factors to facilitate budding in, 350–351
  in genomics, 343–351
  Nef altering cell surface, 348–350
  Tat effects on cellular transcription, 343–348
Viral attachment inhibition
  anti-CD4 antibodies, 97–98
  gp120 envelope
    CD4-binding pocket and CD4 receptor, 96
    cryptic-binding sites and small molecular inhibitors, 97
    cyanovirin-N, 96
    glycan shield, 95
    polyanion compounds, 97
    Retrocyclin-1 and RC-101, 96
Viral CC-class chemokines (vCCLs), 516, 519
Viral entry process
  cellular and viral membranes, fusion of, 1–7
  coreceptor binding, 94
  gp120 attachment to CD4, 94
Viral enzymes, 200–201
Viral FLICE-inhibitory protein (vFLIP), 516–517, 519
Viral gene expression, CDK inhibitor Cyc202 effects on, 330
Viral G protein-coupled receptor (vGPCR), 516–519
Viral IL-6 and IL-10, 515–517, 519
Viral K genes, 516–519
Viral protein-protein interactions
  in genomics, 331–343
  between HIV-1 viral proteins and their cellular-interacting proteins, 331–332
Viral respiratory infections, 485
viral RNA role in HIV genome, 200
Viral targets, in genomics, 329–331
Viread. *See* Tenofovir
Virion proteome approach, in genomics, 351–352
Virucidal strategy of microbicides, 236
Virus replication, NCp7 protein, 239
vpr gene, 330, 384, 412, 425, 431–432, 435, 454, 465–470, 475–476, 480–481, 484
vpu gene, 330, 425, 433, 454, 465–470, 475, 477, 484
vpx gene, 384, 435
V75T mutation and NRTI resistance, 138
Vulva cancers, 528

West Nile Virus, 316
WNV. *See* West Nile Virus

X-linked severe combined immunodeficiency, 307
X-SCID. *See* X-linked severe combined immunodeficiency
X4-tropic viruses, HIV infection, 99

Yeast two-hybrid (Y2H) screens, 332
Y115F mutation and NRTI resistance, 139
Y188L mutation, 148

Zalcitabine and Zidovudine drug role in HIV treatment, 270
Zalcitabine, NRTI, 130
Zidovudine (ZDV), 460–462
  adverse effects, 29–30
  drug interactions, 31
  pharmacokinetics, 29

Contents of Previous Volumes

Volume 40

Advances in Understanding the Pharmacological Properties of Antisense Oligonucleotides
Stanley T. Crooke

Targeted Tumor Cytotoxicity Mediated by Intracellular Single-Chain Anti-Oncogene Antibodies
David T. Curiel

In Vivo Gene Therapy with Adeno-Associated Virus Vectors for Cystic Fibrosis
Terence R. Flotte and Barrie J. Carter

Engineering Herpes Simplex Virus Vectors for Human Gene Therapy
Joseph C. Glorioso, William F. Goins, Martin C. Schmidt, Tom Oligino, Dave Krisky, Peggy Marconi, James D. Cavalcoli, Ramesh Ramakrishnan, P. Luigi Poliani, and David J. Fink

Human Adenovirus Vectors for Gene Transfer into Mammalian Cells
Mary M. Hitt, Christina L. Addison, and Frank L. Graham

Anti-Oncogene Ribozymes for Cancer Gene Therapy
Akira Irie, Hiroshi Kijima, Tsukasa Ohkawa, David Y. Bouffard, Toshiya Suzuki, Lisa D. Curcio, Per Sonne Holm, Alex Sassani, and Kevin J. Scanlon

Cytokine Gene Transduction in the Immunotherapy of Cancer
Giorgio Parmiani, Mario P. Colombo, Cecilia Melani, and Flavio Arienti

Gene Therapy Approaches to Enhance Anti-Tumor Immunity
Daniel L. Shawler, Habib Fakhrai, Charles Van Beveren, Dan Mercoa, Daniel P. Gold, Richard M. Bartholomew, Ivor Royston, and Robert E. Sobol

Modified Steroid Receptors and Steroid-Inducible Promoters as Genetic Switches for Gene Therapy
John H. White

Strategies for Approaching Retinoblastoma Tumor Suppressor Gene Therapy
Hong-Ji Xu

Immunoliposomes for Cancer Treatment
John W. Park, Keelung Hong, Dmitri B. Kirpotin, Demetrios Papahadjopoulos, and Christopher C. Benz

Antisense Inhibition of Virus Infection
R. E. Kilkuskie and A. K. Field

Volume 41

Apoptosis: An Overview of the Process and Its Relevance in Disease
Stephanie Johnson Webb, David J. Harrison, and Andrew H. Wyllie

Genetics of Apoptosis
Serge Desnoyers and Michael O. Hengartner

Methods Utilized in the Study of Apoptosis
Peter W. Mesner and Scott H. Kaufmann

In Vitro Systems for the Study of Apoptosis
Atsushi Takahashi and William C. Earnshaw

The Fas Pathway in Apoptosis
Christine M. Eischen and Paul J. Leibson

Ceramide: A Novel Lipid Mediator of Apoptosis
Miriam J. Smyth, Lina M. Obeid, and Yusuf A. Hannun

Control of Apoptosis by Proteases
Nancy A. Thornberry, Antony Rosen, and Donald W. Nicholson

Death and Dying in the Immune System
David S. Ucker

Control of Apoptosis by Cytokines
W. Stratford May, Jr.

Glucocorticoid-Induced Apoptosis
Clark W. Distelhorst

Apoptosis in AIDS
Andrew D. Badley, David Dockrell, and Carlos V. Paya

Virus-Induced Apoptosis
J. Marie Hardwick

Apoptosis in Neurodegenerative Diseases
Ikuo Nishimoto, Takashi Okamoto, Ugo Giambarella, and Takeshi Iwatsubo

Apoptosis in the Mammalian Kidney: Incidence, Effectors, and Molecular Control in Normal Development and Disease States
Ralph E. Buttyan and Glenda Gobé

Apoptosis in the Heart
Samuil R. Umansky and L. David Tomei

Apoptosis and the Gastrointestinal System
Florencia Que and Gregory J. Gores

Role of *p53* in Apoptosis
Christine E. Canman and Michael B. Kastan

Chemotherapy-Induced Apoptosis
Peter W. Mesner, Jr., I. Imawati Budihardjo, and Scott H. Kaufmann

Bcl-2 Family Proteins: Strategies for Overcoming Chemoresistance in Cancer
John C. Reed

Role of Bcr-Abl Kinase in Resistance to Apoptosis
Afshin Samali, Adrienne M. Gorman, and Thomas G. Cotter

Apoptosis in Hormone-Responsive Malignancies
Samuel R. Denmeade, Diane E. McCloskey, Ingrid B. J. K. Joseph, Hillary A. Hahm, John T. Isaacs, and Nancy E. Davidson

Volume 42

Catecholamine: Bridging Basic Science
Edited by David S. Goldstein, Graeme Eisenhofer, and Richard McCarty

Part A. Catecholamine Synthesis and Release

Part B. Catecholamine Reuptake and Storage

Part C. Catecholamine Metabolism

Part D. Catecholamine Receptors and Signal Transduction

Part E. Catecholamine in the Periphery

Part F. Catecholamine in the Central Nervous System

Part G. Novel Catecholaminergic Systems

Part H. Development and Plasticity

Part I. Drug Abuse and Alcoholism

Volume 43

Overview: Pharmacokinetic Drug–Drug Interactions
Albert P. Li and Malle Jurima-Romet

Role of Cytochrome P450 Enzymes in Drug–Drug Interactions
F. Peter Guengerich

The Liver as a Target for Chemical–Chemical Interactions
John-Michael Sauer, Eric R. Stine, Lhanoo Gunawardhana, Dwayne A. Hill, and I. Glenn Sipes

Application of Human Liver Microsomes in Metabolism-Based Drug–Drug Interactions: *In Vitro–in Vivo* Correlations and the Abbott Laboratories Experience
A. David Rodrigues and Shekman L. Wong

Primary Hepatocyte Cultures as an *in Vitro* Experimental Model for the Evaluation of Pharmacokinetic Drug–Drug Interactions
Albert P. Li

Liver Slices as a Model in Drug Metabolism
James L. Ferrero and Klaus Brendel

Use of cDNA-Expressed Human Cytochrome P450 Enzymes to Study Potential Drug–Drug Interactions
Charles L. Crespi and Bruce W. Penman

Pharmacokinetics of Drug Interactions
Gregory L. Kedderis

Experimental Models for Evaluating Enzyme Induction Potential of New Drug Candidates in Animals and Humans and a Strategy for Their Use
Thomas N. Thompson

Metabolic Drug–Drug Interactions: Perspective from FDA Medical and Clinical Pharmacology Reviewers
John Dikran Balian and Atiqur Rahman

Drug Interactions: Perspectives of the Canadian Drugs Directorate
Malle Jurima-Romet

Overview of Experimental Approaches for Study of Drug Metabolism and Drug–Drug Interactions
Frank J. Gonzalez

Volume 44

Drug Therapy: The Impact of Managed Care
Joseph Hopkins, Shirley Siu, Maureen Cawley, and Peter Rudd

The Role of Phosphodiesterase Enzymes in Allergy and Asthma
D. Spina, L. J. Landells, and C. P. Page

Modulating Protein Kinase C Signal Transduction
Daria Mochly-Rosen and Lawrence M. Kauvar

Preventive Role of Renal Kallikrein—Kinin System in the Early Phase of Hypertension and Development of New Antihypertensive Drugs
Makoto Kartori and Masataka Majima

The Multienzyme PDE4 Cyclic Adenosine Monophosphate-Specific Phosphodiesterase Family: Intracellular Targeting, Regulation, and Selective Inhibition by Compounds Exerting Anti-inflammatory and Antidepressant Actions
Miles D. Houslay, Michael Sullivan, and Graeme B. Bolger

Clinical Pharmacology of Systemic Antifungal Agents: A Comprehensive Review of Agents in Clinical Use, Current Investigational Compounds, and Putative Targets for Antifungal Drug Development
Andreas H. Groll, Stephen C. Piscitelli, and Thomas J. Walsh

Volume 45

Cumulative Subject Index
Volumes 25–44

Volume 46

Therapeutic Strategies Involving the Multidrug Resistance Phenotype: The *MDR1* Gene as Target, Chemoprotectant, and Selectable Marker in Gene Therapy
Josep M. Aran, Ira Pastan, and Michael M. Gottesman

The Diversity of Calcium Channels and Their Regulation in Epithelial Cells
Min I. N. Zhang and Roger G. O'Neil

Gene Therapy and Vascular Disease
Melina Kibbe, Timothy Billiar, and Edith Tzeng

Heparin in Inflammation: Potential Therapeutic Applications beyond Anticoagulation
David J. Tyrrell, Angela P. Horne, Kevin R. Holme, Janet M. H. Preuss, and Clive P. Page

The Regulation of Epithelial Cell cAMP- and Calcium-Dependent Chloride Channels
Andrew P. Morris

Calcium Channel Blockers: Current Controversies and Basic Mechanisms of Action
William T. Clusin and Mark E. Anderson

Mechanisms of Antithrombotic Drugs
Perumal Thiagarajan and Kenneth K. Wu

Volume 47

Hormones and Signaling
Edited by Bert W. O'Malley

New Insights into Glucocorticoid and Mineralocorticoid Signaling: Lessons from Gene Targeting
Holger M. Reichardt, François Tronche, Stefan Berger, Christoph Kellendonk, and Günther Shütz

Orphan Nuclear Receptors: An Emerging Family of Metabolic Regulators
Robert Sladek and Vincent Giguère

Nuclear Receptor Coactivators
Stefan Westin, Michael G. Rosenfeld, and Christopher K. Glass

Cytokines and STAT Signaling
Christian Schindler and Inga Strehlow

Coordination of cAMP Signaling Events through PKA Anchoring
John D. Scott, Mark L. Dell'Acqua, Iain D. C. Fraser, Steven J. Tavalin, and Linda B. Lester

G Protein-Coupled Extracellular Ca^{2+} (Ca^{2+}_o)-Sensing Receptor (CaR): Roles in Cell Signaling and Control of Diverse Cellular Functions
Toru Yamaguchi, Naibedya Chattopadhyay, and Edward M. Brown

Pancreatic Islet Development
Debra E. Bramblett, Hsiang-Po Huang, and Ming-Jer Tsai

Genetic Analysis of Androgen Receptors in Development and Disease
A. O. Brinkmann and J. Trapman

An Antiprogestin Regulable Gene Switch for Induction of Gene Expression *in Vivo*
Yaolin Wang, Sophia Y. Tsai, and Bert W. O'Malley

Steroid Receptor Knockout Models: Phenotypes and Responses Illustrate Interactions between Receptor Signaling Pathways *in Vivo*
Sylvia Hewitt Curtis and Kenneth S. Korach

Volume 48

HIV: Molecular Biology and Pathogenesis: Viral Mechanisms
Edited by Kuan-Teh Jeang

Multiple Biological Roles Associated with the Repeat (R) Region of the HIV-I RNA Genome
Ben Berkhout

HIV Accessory Proteins: Multifunctional Components of a Complex System
Stephan Bour and Klaus Strebel

Role of Chromatin in HIV-I Transcriptional Regulation
Carine Van Lint

NF-κB and HIV: Linking Viral and Immune Activation
Arnold B. Rabson and Hsin-Ching Lin

Tat as a Transcriptional Activator and a Potential Therapeutic Target for HIV-1
Anne Gatignol and Kuan-Teh Jeang

From the Outside In: Extracellular Activities of HIV Tat
Douglas Noonan and Andriana Albini

Rev Protein and Its Cellular Partners
Jørgen Kjems and Peter Askjaer

HIV-I Nef: A Critical Factor in Viral-Induced Pathogenesis
A. L. Greenway, G. Holloway, and D. A. McPhee

Nucleocapsid Protein of Human Immunodeficiency Virus as a Model Protein with Chaperoning Functions and as a Target for Antiviral Drugs
Jean-Luc Darlix, Gaël Cristofari, Michael Rau, Christine Péchoux, Lionel Berthoux, and Bernard Roques

Bioactive CD4 Ligands as Pre- and/or Postbinding Inhibitors of HIV-I
Laurence Briant and Christian Devaux

Coreceptors for Human Immunodeficiency Virus and Simian Immunodeficiency Virus
Keith W. C. Peden and Joshua M. Farber

Volume 49

HIV: Molecular Biology and Pathogenesis: Clinical Applications
Edited by Kuan-Teh Jeang

Inhibitors of HIV-I Reverse Transcriptase
Michael A. Parniak and Nicolas Sluis-Cremer

HIV-I Protease: Maturation, Enzyme Specificity, and Drug Resistance
John M. Louis, Irene T. Weber, József Tözsér, G. Marius Clore, and Angela M. Gronenborn

HIV-I Integrase Inhibitors: Past, Present, and Future
Nouri Neamati, Christophe Marchand, and Yves Pommier

Selection of HIV Replication Inhibitors: Chemistry and Biology
Seongwoo Hwang, Natarajan Tamilarasu, and Tariq M. Rana

Therapies Directed against the Rev Axis of HIV Autoregulation
Andrew I. Dayton and Ming Jie Zhang

HIV-I Gene Therapy: Promise for the Future
Ralph Dornburg and Roger J. Pomerantz

Assessment of HIV Vaccine Development: Past, Present, and Future
Michael W. Cho

HIV-I-Associated Central Nervous System Dysfunction
Fred C. Krebs, Heather Ross, John McAllister, and Brian Wigdahl

Molecular Mechanisms of Human Immunodeficiency Virus Type I Mother-Infant Transmission
Nafees Ahmad

Molecular Epidemiology of HIV-I: An Example of Asia
Mao-Yuan Chen and Chun-Nan Lee

Simian Immunodeficiency Virus Infection of Monkeys as a Model System for the Study of AIDS Pathogenesis, Treatment, and Prevention
Vanessa M. Hirsch and Jeffrey D. Lifson

Animal Models for AIDS Pathogenesis
John J. Trimble, Janelle R. Salkowitz, and Harry W. Kestler

Volume 50

General Introduction to Vasopressin and Oxytocin: Structure/Metabolism, Evolutionary Aspects, Neural Pathway/Receptor Distribution, and Functional Aspects Relevant to Memory Processing
Barbara B. McEwen

De Wied and Colleagues I: Evidence for a VP and an OT Influence on MP: Launching the "VP/OT Central Memory Theory"
Barbara B. McEwen

De Wied and Colleagues II: Further Clarification of the Roles of Vasopressin and Oxytocin in Memory Processing
Barbara B. McEwen

De Wied and Colleagues III: Brain Sites and Transmitter Systems Involved in theVasopressin and Oxytocin Influence on Memory Processing
Barbara B. McEwen

De Wied and Colleagues IV: Research into Mechanisms of Action by Which Vasopressin and Oxytocin Influence Memory Processing
Barbara B. McEwen

Research Studies of Koob and Colleagues: The "Vasopressin Dual Action Theory"
Barbara B. McEwen

Contributions of Sahgal and Colleagues: The "Vasopression Central Arousal Theory"
Barbara B. McEwen

Role of Attentional Processing in Mediating the Influence of Vasopressin on Memory Processing
Barbara B. McEwen

Expansion of Vasopressin/Oxytocin Memory Research I: Peripheral Administration
Barbara B. McEwen

Expansion of Vasopressin/Oxytocin Memory Research II: Brain Structures and Transmitter Systems Involved in the Influence of Vasopressin and Oxytocin on Memory Processing
Barbara B. McEwen

Expansion of Vasopressin/Oxytocin Memory Research III: Research Summary and Commentary on Theoretical and Methodological Issues
Barbara B. McEwen

Research Contributions of Dantzer, Bluthe, and Colleagues to the Study of the Role of Vasopressin in Olfactory-Based Social Recognition Memory
Barbara B. McEwen

Expansion of Olfactory-Based Social Recognition Memory Research: The Roles of Vasopressin and Oxytocin in Social Recognition Memory
Barbara B. McEwen

Brain–Fluid Barriers: Relevance for Theoretical Controversies Regarding Vasopressin and Oxytocin Memory Research
Barbara B. McEwen

Closing Remarks: Review and Commentary on Selected Aspects of the Roles of Vasopressin and Oxytocin in Memory Processing
Barbara B. McEwen

Volume 51

Treatment of Leukemia and Lymphoma
Edited by David A. Scheinberg and Joseph G. Jurcic

Kinase Inhibitors in Leukemia
Mark Levis and Donald Small

Therapy of Acute Promyelocytic Leukemia
Steven Soignet and Peter Maslak

Investigational Agents in Myeloid Disorders
Farhad Ravandi and Jorge Cortes

Methodologic Issues in Investigation of Targeted Therapies in Acute Myeloid Leukemia
Elihu Estey

Purine Analogs in Leukemia
Nicole Lamanna and Mark Weiss

Monoclonal Antibody Therapy in Lymphoid Leukemias
Thomas S. Lin and John C. Byrd

Native Antibody and Antibody-Targeted Chemotherapy for Acute Myeloid Leukemia
Eric L. Sievers

Radioimmunotherapy of Leukemia
John M. Burke and Joseph G. Jurcic

Immunotoxins and Toxin Constructs in the Treatment of Leukemia and Lymphoma
Michael Rosenblum

Antibody Therapy of Lymphoma
George J. Weiner and Brian K. Link

Vaccines in Leukemia
Sijie Lu, Eric Wieder, Krishna Komanduri, Qing Ma, and Jeffrey J. Molldrem

Therapeutic Idiotype Vaccines for Non-Hodgkin's Lymphoma
John M. Timmerman

Cytokine Modulation of the Innate Immune System in the Treatment of Leukemia and Lymphoma
Sherif S. Farag and Michael A. Caligiuri

Donor Lymphocyte Infusions
Vincent T. Ho and Edwin P. Alyea

Somatic Cell Engineering and the Immunotherapy of Leukemias and Lymphomas
Renier J. Brentjens and Michel Sadelain

Volume 52

Historical Background
Andrew Young

Tissue Expression and Secretion of Amylin
Andrew Young

Receptor Pharmacology
Andrew Young

Amylin and the Integrated Control of Nutrient Influx
Andrew Young

Inhibition of Food Intake
Andrew Young

Inhibition of Gastric Emptying
Andrew Young

Effects on Digestive Secretions
Andrew Young

Inhibition of Glucagon Secretion
Andrew Young

Inhibition of Insulin Secretion
Andrew Young

Effects on Plasma Glucose and Lactate
Andrew Young

Effects in Skeletal Muscle
Andrew Young

Effects in Liver
Andrew Young

Effects in Fat
Andrew Young

Cardiovascular Effects
Andrew Young

Renal Effects
Andrew Young

Effects on Bone
Andrew Young

Central Nervous System and Other Effects
Andrew Young

Clinical Studies
Andrew Young

Volume 53

Isolation, Purification, and Analysis of Chondroitin Sulfate Proteoglycans
Fumiko Matsui and Atsuhiko Oohira

Isolation and Purification of Chondroitin Sulfate
Luiz-Claudio F. Silva

Structure of Chondroitin Sulfate
Fotini N. Lamari and Nikos K. Karamanos

Progress in the Structural Biology of Chondroitin Sulfate
Barbara Mulloy

The Biosynthesis and Catabolism of Galactosaminoglycans
Vikas Prabhakar and Ram Sasisekharan

Biosynthesis of Chondroitin Sulfate: From the Early, Precursor Discoveries to Nowadays, Genetics Approaches
Mauro S. G. Pavão, Ana Cristina Vilela-Silva, and Paulo A. S. Mourão

Advances in the Analysis of Chondroitin/Dermatan Sulfate
M. Stylianou, I.-E. Triantaphyllidou, and D. H. Vynios

Chondroitin Sulfate Lyases: Applications in Analysis and Glycobiology
Emmanuel Petit, Cedric Delattre, Dulce Papy-Garcia, and Philippe Michaud

CS Lyases: Structure, Activity, and Applications in Analysis and the Treatment of Diseases
Robert J. Linhardt, Fikri Y. Avci, Toshihiko Toida, Yeong Shik Kim, and Miroslaw Cygler

Structure, Metabolism, and Tissue Roles of Chondroitin Sulfate Proteoglycans
Christopher J. Handley, Tom Samiric, and Mirna Z. Ilic

Emergence and Structural Characteristics of Chondroitin Sulfates in the Animal Kingdom
Lucia O. Sampaio and Helena B. Nader

Role of the Sulfation Pattern of Chondroitin Sulfate in its Biological Activities and in the Binding of Growth Factors
Chilkunda D. Nandini and Kazuyuki Sugahara

Chondroitin Sulfate as a Key Molecule in the Development of Atherosclerosis and Cancer Progression
A. D. Theocharis, I. Tsolakis, G. N. Tzanakakis, and N. K. Karamanos

Chondroitin Sulfate Proteoglycans in Tumor Progression
Yanusz Wegrowski and François-Xavier Maquart

Chondroitin Sulfate Proteoglycans in the Brain
Sachiko Aono and Atsuhiko Oohira

Chondroitin/Dermatan Sulfates in the Central Nervous System: Their Structures and Functions in Health and Disease
Uwe Rauch and Joachim Kappler

Chondroitin Sulfate Proteoglycan and its Degradation Products in CNS Repair
Asya Rolls and Michal Schwartz

Role of Chondroitin-4-Sulfate in Pregnancy-Associated Malaria
D. Channe Gowda

Immunological Activity of Chondroitin Sulfate
Toshihiko Toida, Shinobu Sakai, Hiroshi Akiyama, and Robert J. Linhardt

Antioxidant Activity of Chondroitin Sulfate
G. M. Campo, A. Avenoso, S. Campo, A. M. Ferlazzo, and A. Calatroni

Effects of Chondroitin Sulfate on the Cellular Metabolism
N. Brandl, J. Holzmann, R. Schabus, and M. Huettinger

In Vitro Effects of Chondroitin Sulfate
A. Fioravanti, R. Marcolongo, and G. Collodel

Effect of Chondroitin Sulfate as Nutraceutical in Dogs with Arthropathies
Britta Dobenecker

Chondroitin Sulfate as a Structure-Modifying Agent
Daniel Uebelhart, Ruud Knols, Eling D de Bruin, and Gust Verbruggen

Chondroitin Sulfate in the Management of Erosive Osteoarthritis of the Interphalangeal Finger Joints
Gust Verbruggen

Chondroitin Sulfate in the Management of Hip and Knee Osteoarthritis: An Overview
Géraldine Bana, Bénédicte Jamard, Evelyne Verrouil, and Bernard Mazières

Treatment of Knee Osteoarthritis with Oral Chondroitin Sulfate
Daniel Uebelhart, Ruud Knols, Eling D de Bruin, and Gust Verbruggen

Volume 54

The Role of GABA in the Mediation and Perception of Pain
S. J. Enna and Kenneth E. McCarson

Distribution of GABA Receptors in the Thalamus and Their Involvement in Nociception
Fani L. Neto, Joana Ferreira-Gomes, and José M. Castro-Lopes

$GABA_A$ Agonists and Partial Agonists: THIP (Gaboxadol) as a Non-Opioid Analgesic and a Novel Type of Hypnotic
Povl Krogsgaard-Larsen, Bente Frølund, and Tommy Liljefors

Rat Modeling for GABA Defects in Schizophrenia
Francine M. Benes and Barbara Gisabella

Epigenetic Targets in GABAergic Neurons to Treat Schizophrenia
E. Costa, E. Dong, D. R. Grayson, W. B. Ruzicka, M. V. Simonini, M. Veldic, and A. Guidotti

GABAergic Malfunction in the Limbic System Resulting from an Aboriginal Genetic Defect in Voltage-Gated Na^+-Channel SCN5A is Proposed to Give Rise to Susceptibility to Schizophrenia
Eugene Roberts

$GABA_A$ Receptor Mutations Associated with Generalized Epilepsies
Robert L. Macdonald, Jing-Qiong Kang, Martin J. Gallagher, and Hua-Jun Feng

From Gene to Behavior and Back Again: New Perspectives on $GABA_A$ Receptor Subunit Selectivity of Alcohol Actions
Stephen L. Boehm II, Igor Ponomarev, Yuri A. Blednov, and R. Adron Harris

A Role for GABA in Alcohol Dependence
George F. Koob

Structure, Pharmacology, and Function of $GABA_A$ Receptor Subtypes
Werner Sieghart

Structure–Activity Relationship and Pharmacology of γ-Aminobutyric Acid (GABA) Transport Inhibitors
Rasmus Prætorius Clausen, Karsten Madsen, Orla Miller Larsson, Bente Frølund, Povl Krogsgaard-Larsen, and Arne Schousboe

Modulation of Ionotropic GABA Receptors by Natural Products of Plant Origin
Graham A. R. Johnston, Jane R. Hanrahan, Mary Chebib, Rujee K. Duke, and Kenneth N. Mewett

Volume 55

HIV-1 RNA Packaging
Andrew M. L. Lever

Structure and Function of the HIV Envelope Glycoprotein as Entry Mediator, Vaccine Immunogen, and Target for Inhibitors
Ponraj Prabakaran, Antony S. Dimitrov, Timothy R. Fouts, and Dimiter S. Dimitrov

HIV-1 Reverse Transcription: Close Encounters Between the Viral Genome and a Cellular tRNA
Truus E. M. Abbink and Ben Berkhout

Transcription of HIV: Tat and Cellular Chromatin
Anne Gatignol

Posttranscriptional Control of HIV-1 and Other Retroviruses and Its Practical Applications
Barbara K. Felber, Andrei S. Zolotukhin, and George N. Pavlakis

HIV Accessory Genes Vif and Vpu
Klaus Strebel

Interactions of HIV-1 Viral Protein R with Host Cell Proteins
Richard Y. Zhao, Robert T. Elder, and Michael Bukrinsky

HIV-1 Protease: Structure, Dynamics, and Inhibition
John M. Louis, Rieko Ishima, Dennis A. Torchia, and Irene T. Weber

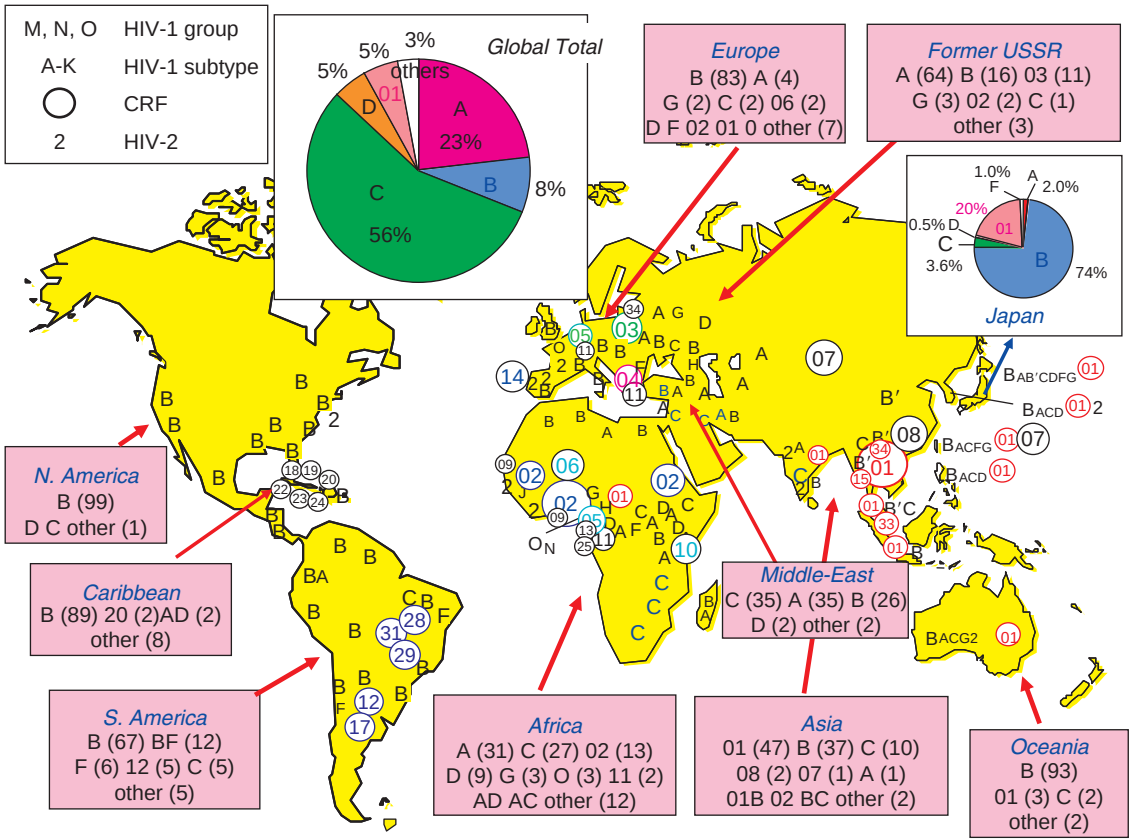
Properties, Functions, and Drug Targeting of the Multifunctional Nucleocapsid Protein of the *Human Immunodeficiency Virus*
Jean-Luc Darlix, José Luis Garrido, Nelly Morellet, Yves Mély, and Hugues de Rocquigny

Human Immunodeficiency Virus Type 1 Assembly, Release, and Maturation
Catherine S. Adamson and Eric O. Freed

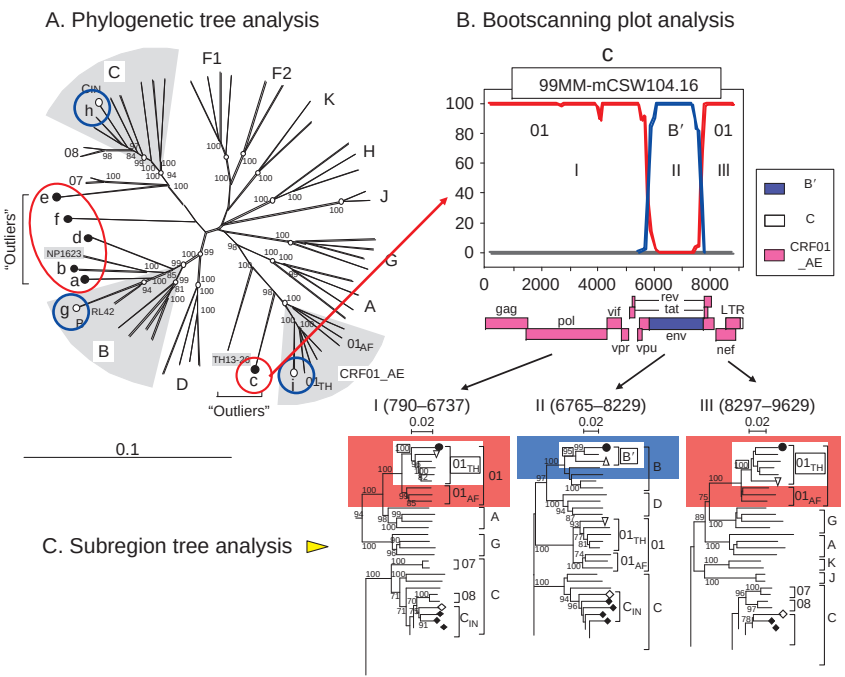
Role of Nef in HIV-1 Replication and Pathogenesis
John L. Foster and J. Victor Garcia

Treatment Implications of the Latent Reservoir for HIV-1
Susan Peterson, Alison P. Reid, Scott Kim, and Robert F. Siliciano

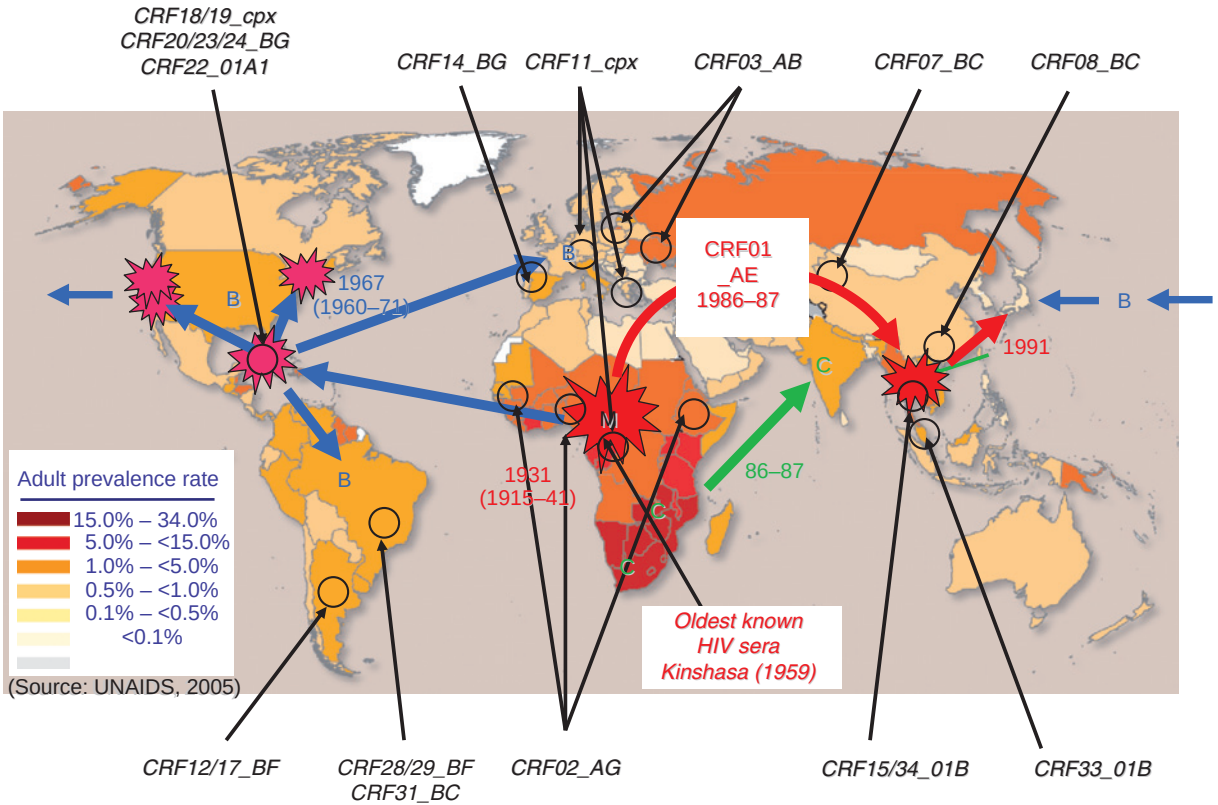
RNA Interference and HIV-1
Man Lung Yeung, Yamina Bennasser, Shu-Yun Le, and Kuan-Teh Jeang



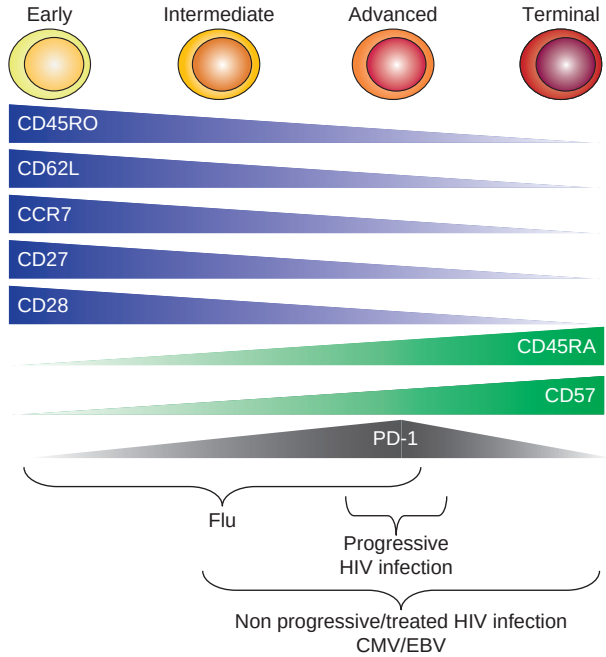
CHAPTER 1, FIGURE 4 Global distribution of HIV genotypes and their estimated proportions. The number in parenthesis after each genotype is the proportion (in percentage) of the indicated genotype in the respective geographic regions. Data source (http://www.hiv.lanl.gov/components/hiv-web/new_geography/). A global total is adopted from Esparza and Bhamarapravati (2000). HIV-1 genotype distribution in Japan is also included.


A. Phylogenetic tree analysis
B. Bootscanning plot analysis
C. Subregion tree analysis
C
99MM-mCSW104.16
100
80
60
40
20
0
0
2000
4000
6000
8000
01
B′
01
I
II
III
B′
C
CRF01
_AE
gag
pol
vif
rev
tat
LTR
vpr
vpu
env
nef
I (790–6737)
II (6765–8229)
III (8297–9629)
0.02
0.1
F1
F2
K
H
J
G
A
01AF
CRF01_AE
01TH
C
i
c
TH13-26
D
B
"Outliers"
"Outliers"
C
h
08
07
e
f
d
NP1623
b
a
g
RL42
01TH
01AF
A
G
07
08
C
CIN
B
B′
D
01
C
CIN
G
A
K
J
C

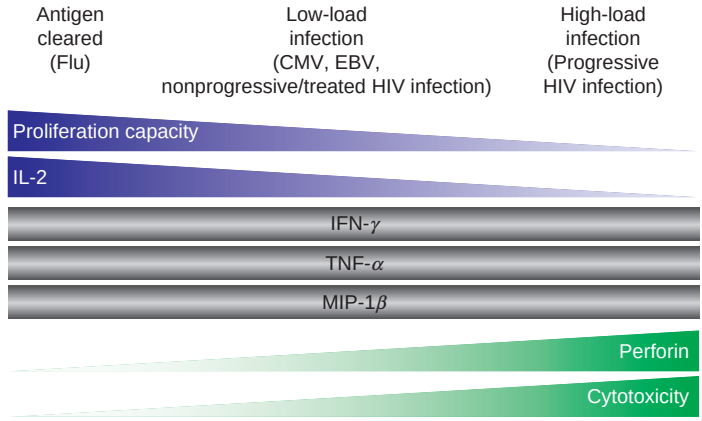
CHAPTER 1, FIGURE 5 Phylogenetic tree analysis (A) and recombination breakpoint analysis for identification novel HIV-1 recombinant strains. Neighbor-joining tree analysis of Myanmar HIV-1 isolates based on near full-length sequences. Strains (g–i) belong to nonrecombinant forms of HIV-1 subtypes B′ (Thailand variant of subtype B) and C, and CRF01_AE, respectively. Strains (a–f) are "outliers" that are not assigned to any known HIV-1 genotypes (subtypes/CRFs). They turned out to be novel HIV-1 intergenotype recombinants, that is, "unique recombinant forms" (URFs). The data outputs obtained from various recombination breakpoint analyses, including bootscanning plot analysis (B) and subregion tree analysis (C) for "outlier" strain (c) are schematically illustrated. The results indicate that the strain (c) is a novel HIV-1 URF composed of subtype B′ and CRF01_AE of Thailand origins. The strain (c) shows the structural similarity to CRF15_01B, but is not exactly identical (Takebe *et al.*, 2003).



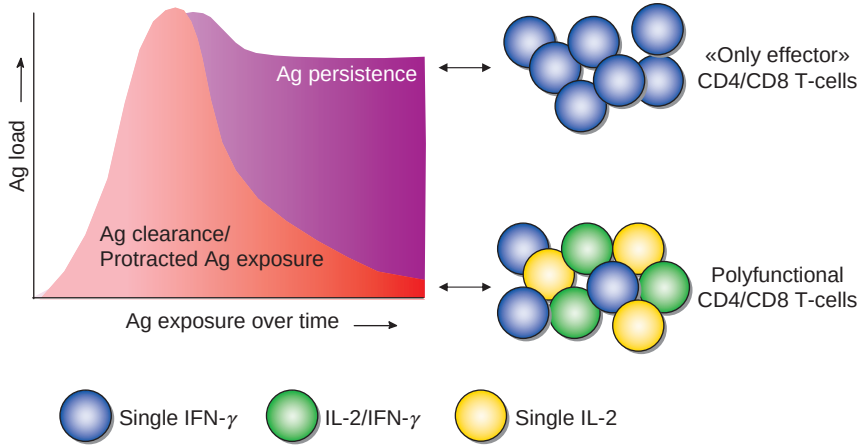
CHAPTER 1, FIGURE 6 Origin of HIV-1 group M and plausible routes of the spread of the HIV-1 strains responsible for epidemic in Asia. The epidemic focuses of selected CRFs of geographical relevance are shown. The illustrations are superimposed on the world map with estimated adult HIV prevalence in different countries (UNAIDS/WHO, 2005).



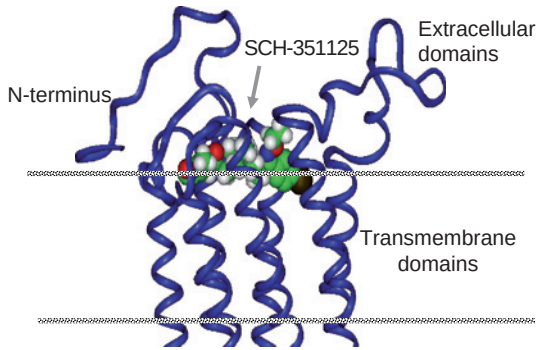
CHAPTER 3, FIGURE 1 Relationship between phenotypic markers, stages of differentiation, and phenotype of virus-specific CD4/8 T cells. Early stage differentiation is associated with the expression of the majority of markers. Differentiation is associated with the progressive loss of a large number of markers. Expression of CD45RA and CD57 in the absence of the other markers defines a terminal differentiation stage.



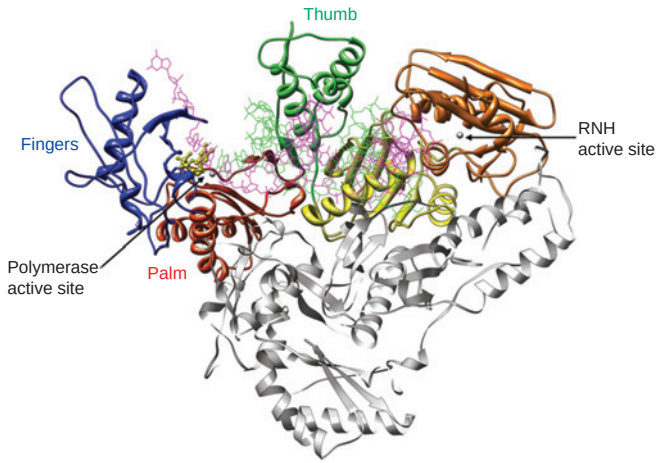
CHAPTER 3, FIGURE 2 Schematic representation of the functional profile of virus-specific CD4 and CD8 T cells based on the level/duration of antigen exposure/load. All functions are relevant for both CD4 and CD8 T cells with the exception of perforin expression and cytotoxicity which pertain to CD8 T cells.



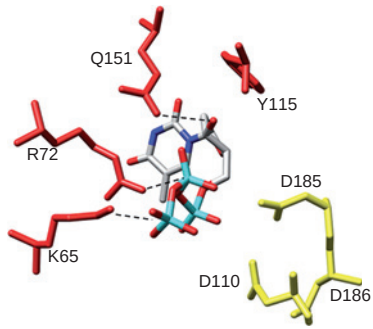
CHAPTER 3, FIGURE 3 Association between the level of antigen exposure and the functional profile of virus-specific CD4 and CD8 T cells. Single IFN-γ and dual IFN-γ/IL-2 are relevant for both CD4 and CD8 T cells while single IL-2 secreting T cells are only considered for CD4 T cells.



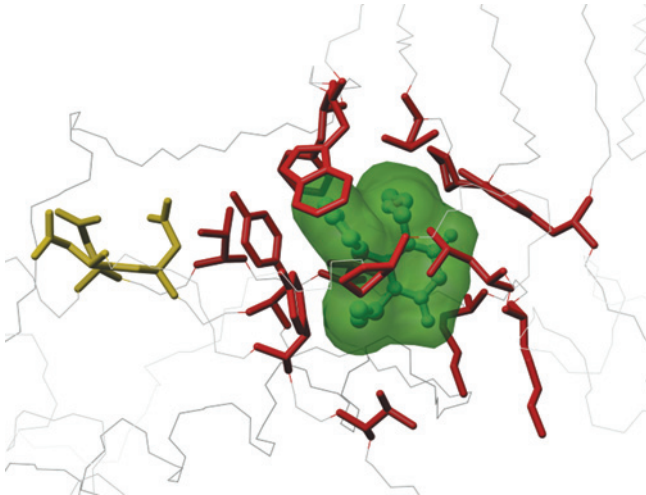
CHAPTER 4, FIGURE 3 Model of a small molecule binding to CCR5. A model of CCR5 was generated based on the crystal structure of bacterial rhodopsin and a putative binding site for the small molecule antagonist SCH-351125 was modeled on the receptor based on mutagenesis and binding studies. Small molecules, such as SCH 351125, bind within a hydrophobic pocket formed within the membrane near the surface of the cells by the transmembrane domains and proximal extracellular loop regions. Mapping studies have demonstrated that individual small molecules contact different residues within the pocket and extracellular loops. This differential binding can account for different activities of the compounds on the receptor.



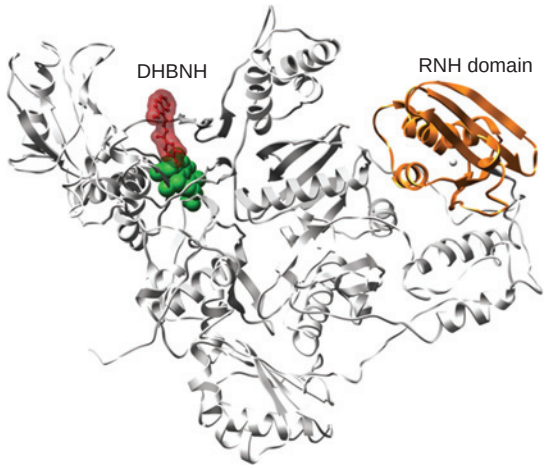
CHAPTER 5, FIGURE 1 Structure of the HIV-1 reverse transcriptase (RT) p66/p51 heterodimer. The p66 subunit is rendered in color while the p51 subunit is in gray. The various subdomains of the p66 subunit are depicted in different colors: fingers (blue), palm (red), thumb (green), connection (yellow), and ribonuclease H (orange). The template strand of the bound nucleic acid is in magenta while the primer strand is green. Relative locations of the DNA polymerase and RNH active sites are indicated. The figure is based on pdb file 1RTD (Huang *et al*., 1998) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al*., 2004).



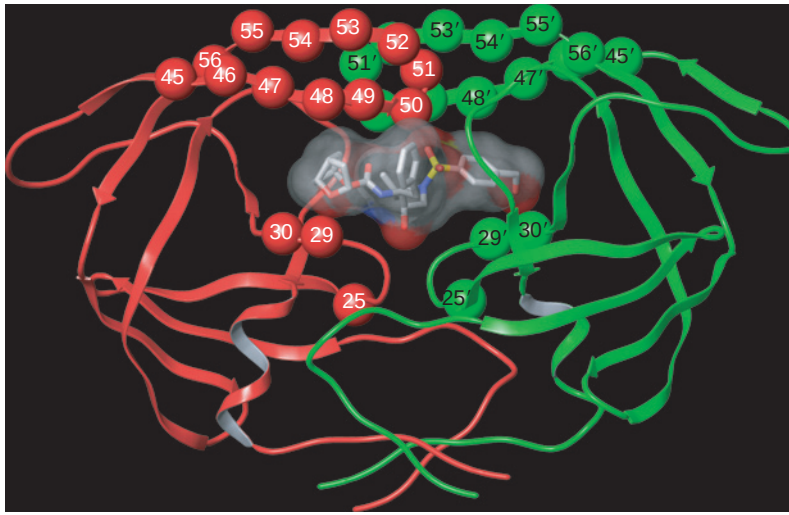
CHAPTER 5, FIGURE 2 Relative positions of essential RT polymerase active site residues. The catalytic aspartates D110, D185, and D186 are in yellow, and the residues making important contacts with the bound dNTP (center) are in red. The figure is derived from pdb file 1RTD (Huang *et al*., 1998) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al*., 2004).



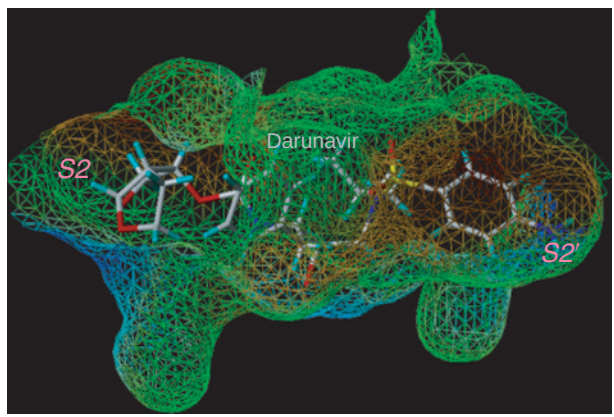
CHAPTER 5, FIGURE 6 Location of the nonnucleoside inhibitor binding pocket (NNIBP) relative to the RT DNA polymerase active site. RT polymerase catalytic aspartates D110, D185, and D186 are rendered in yellow. The NNIBP is defined by the bound NNRTI (green) surrounded by amino acid residues that form the NNIBP (red). Details are provided in the text. The figure is derived from pdb file 1RT1 (Ren *et al.*, 1995) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004).



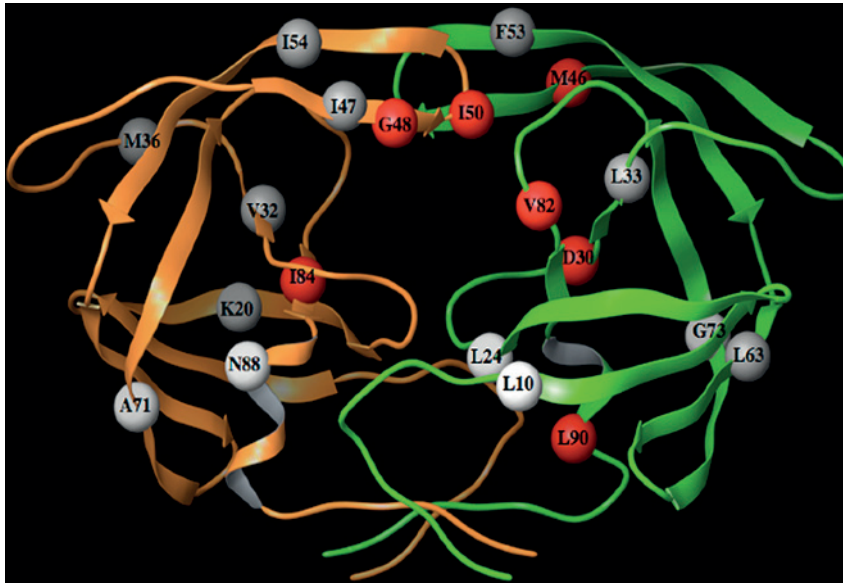
CHAPTER 5, FIGURE 9 Location of the ribonuclease H (RNH)-specific inhibitor dihydroxy benzoyl naphthyl hydrazone (DHBNH) relative to the RNH domain of HIV-1 RT. DHBNH is rendered in red spacefill. The nonnucleoside inhibitor binding pocket (NNIBP) is indicated by residues in green spacefill. The figure is derived from pdb file 215J (Himmel *et al.*, 2006) and was drawn using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004).



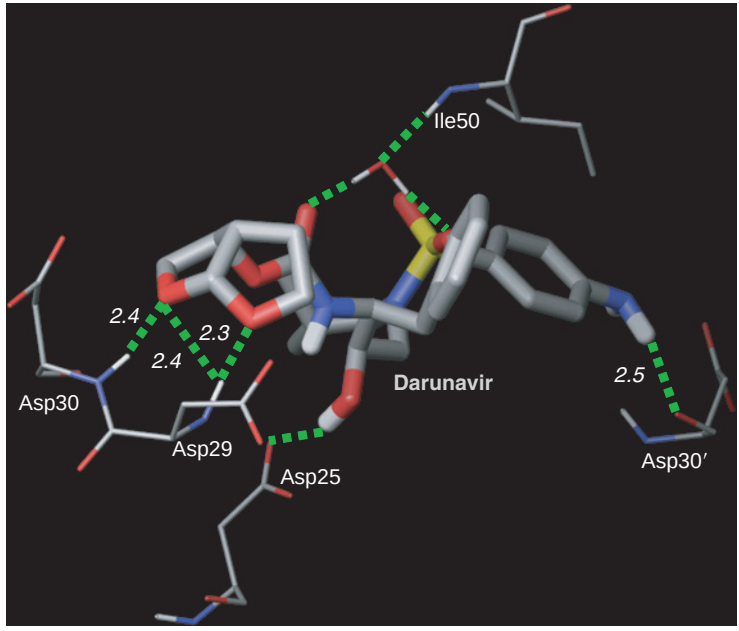
CHAPTER 6, FIGURE 1 Structure of HIV-1 protease. The HIV protease consists of two identical 99 amino acid subunits and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′). Each monomer contributes amino acids (positions 45–56) to form a flap that extends over the substrate-binding cleft. The active site is covered over by two β-hairpin structures or "flaps" that are highly flexible and undergo large localized conformational changes during the binding and release of inhibitors and substrates.



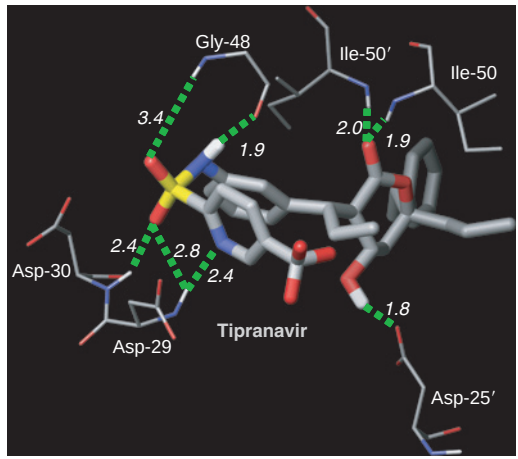
CHAPTER 6, FIGURE 3 Darunavir bound in the hydrophobic cavity within protease. Hydrophobic cavity within protease with darunavir (DRV/TMC114; PDB ID 1S6G) is shown. Brown and green regions are lipophilic while the blue regions are hydrophilic (determined using MOLCAD). The S2 and S2′ subsites are indicated. The figure was generated using Sybyl 7.0 (Tripos, Inc.).



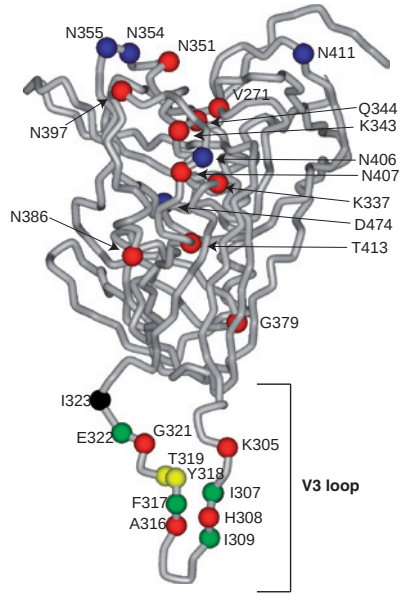
CHAPTER 6, FIGURE 4 Locations of amino acid substitutions associated with drug resistance to PIs. Structure of protease homodimer with positions of amino acid residues associated with clinical resistance to current PIs is indicated. Primary and secondary mutations are indicated with red and white spheres, respectively. The protease monomers are shown in green and orange ribbons. Mutations are shown on only one monomer for clarity. The figure was generated using Maestro version 7.5.



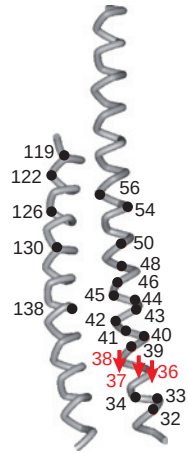
CHAPTER 6, FIGURE 5 Hydrogen bond interactions of darunavir with protease catalytic sites. Hydrogen bond interactions of darunavir with Asp-29 and Asp-30 in the S2 subsite, and Asp-30′ in the S2′ subsite. The hydrogen bonds are shown in green broken lines. The figure was generated using Maestro version 7.5.



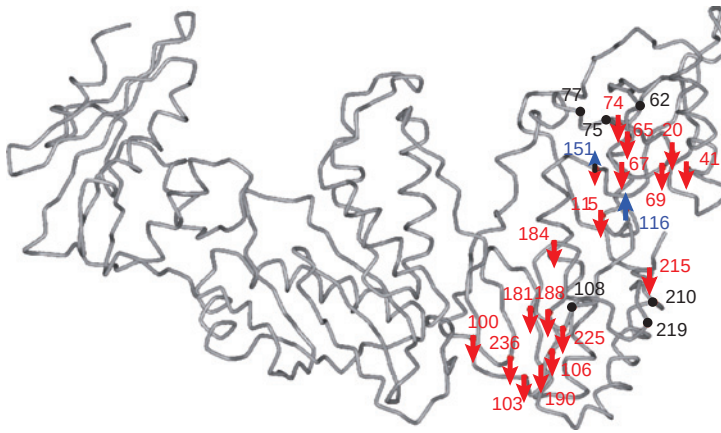
CHAPTER 6, FIGURE 6 Hydrogen bond interactions of TPV with protease. Hydrogen bond interactions of TPV with Asp-29 and Asp-30 in the S2 subsite, with a catalytic aspartate (Asp-25′), and with flap residues Gly-48, Ile-50, and Ile-50′. The hydrogen bonds are shown in green dashed lines. The figure was generated using Maestro version 7.5.



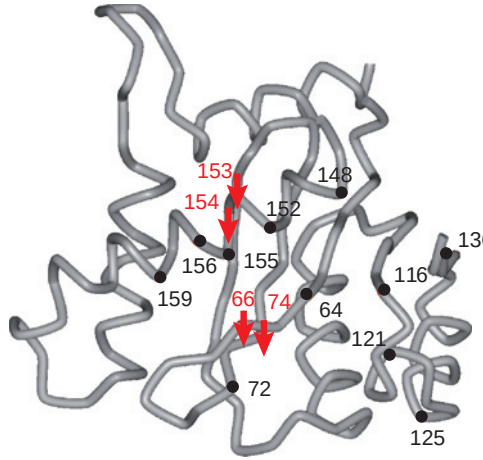
CHAPTER 9, FIGURE 1 Location of amino acid substitutions in the Env gp120 crystal structure selected by *in vitro* passage with CCR5 antagonists. Unlike the primary resistance mutations directly associated with RTIs, PIs, and INIs, HIV-1 appears to follow different evolutionary pathways to resistance dependent on the *env* sequence and CCR5 or CXCR4 antagonist. The amino acids in the HIV-1 Env gp120 structure (Huang *et al*., 2005) selected or associated with resistance to AD101 are shown in red, SCH-D—blue, Tak-779—green, RANTES—yellow, and maraviroc—black. As described in the text, studies have not fully confirmed which mutations are responsible for primary resistance and which are secondary or compensatory.



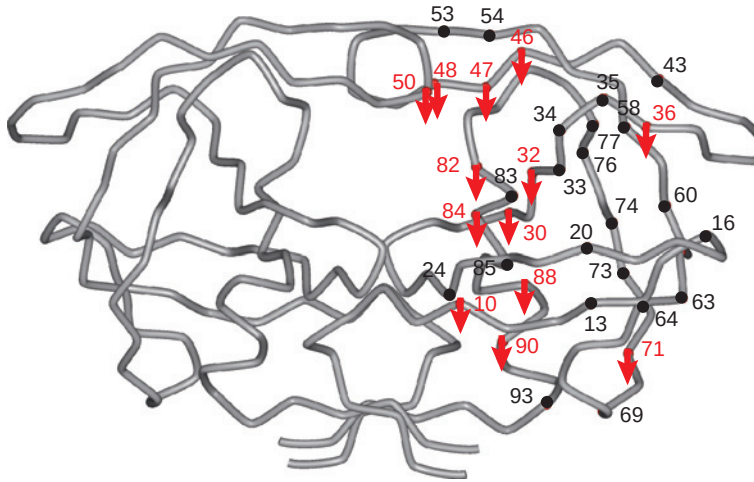
CHAPTER 9, FIGURE 2 Location of amino acid substitutions in the Env gp41 crystal structure selected during treatment or *in vitro* passage with fusion inhibitors. The crystal structure depicts two facing alpha helices from the HR1 and HR2 domains within the six alpha helix bundle (Chan *et al*., 1997). The mutations associated with resistance to ENF, C34, and N44* peptides are labeled. A black circle identifies mutations selected under drug pressure but without a tested effect on replicative fitness. A gray arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.



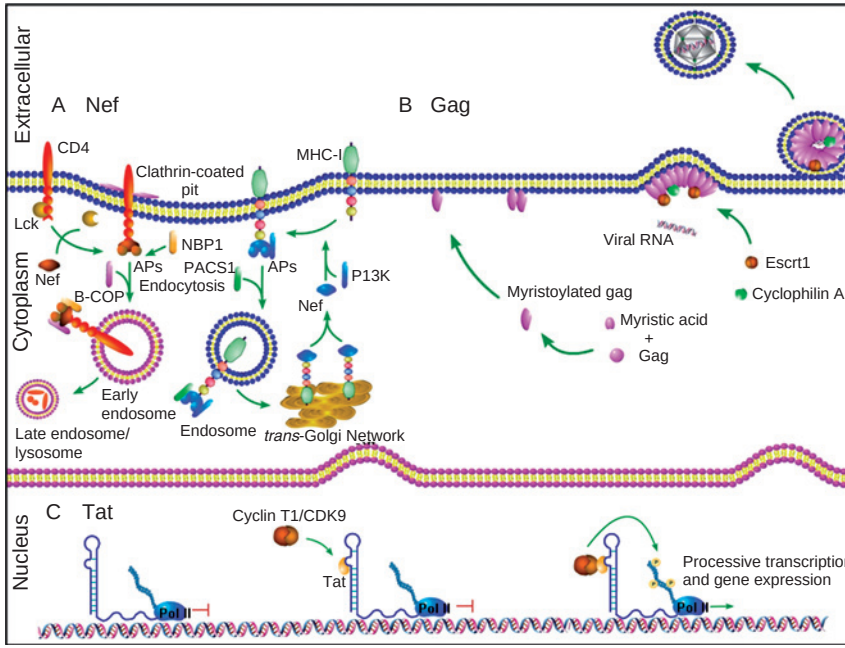
CHAPTER 9, FIGURE 3 Location of RTI resistance mutations in the HIV-1 RT crystal structure. All of the known drug resistance mutations conferring resistance to NRTIs and NNRTIs are mapped onto this RT crystal structure (Rodgers *et al.*, 1995). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness. A blue arrow signifies a drug resistance mutation associated with an increase in replicative fitness.



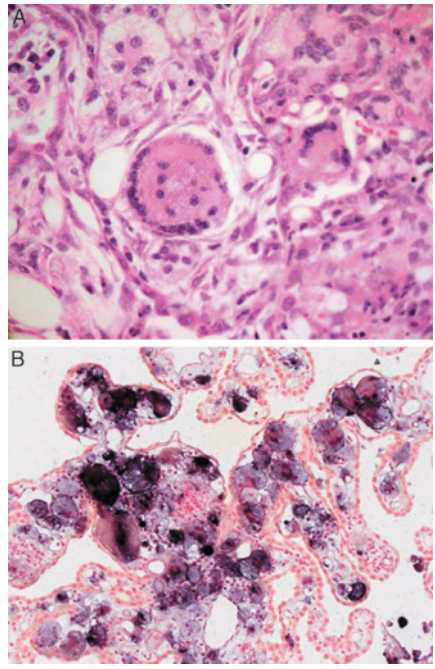
CHAPTER 9, FIGURE 4 Location of INI resistance mutations in the IN core crystal structure. The mutations selected during *in vitro* passage with INIs or selected during treatment with MK-0518 are mapped onto the IN core crystal structure (Goldgur *et al*., 1998). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.



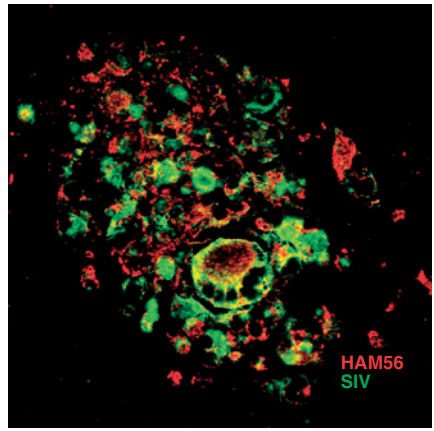
CHAPTER 9, FIGURE 5 Location of PI resistance mutations in the protease crystal structure. The PI resistance mutations were mapped onto the PR core crystal structure (Wlodawer *et al*., 1989). A black circle indicates the position of selected amino acid with a neutral or untested replicative fitness. A red arrow identifies the location of the drug resistance mutations and signifies a decrease in replicative fitness.



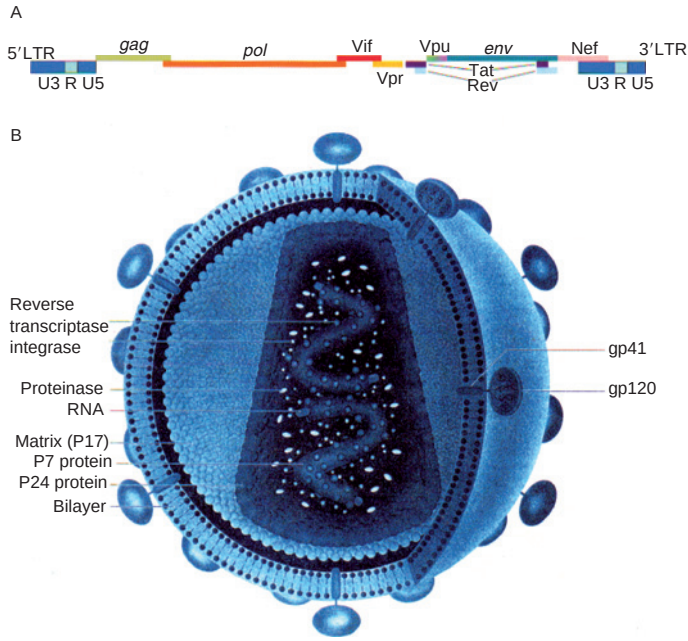
CHAPTER 11, FIGURE 1 Alteration and subversion of cellular processes by *human immunodeficiency virus 1* (HIV-1). (A) Nef removes CD4 and major histocompatibility complex (MHC) from the cell surface by endocytosis and degradation. Nef competitively binds the C-terminal cytoplasmic tail of CD4, displacing bound Lck. AP allow binding of β-COP1, which binds to CD4-bound Nef, facilitated by binding of Nef-binding protein-1 (NBP1). β-COP1 then drives formation of the early endosome and finally the Nef complex dissociates from CD4 as it is further degraded in the lysosome. For MHC, binding of Nef to PI3K allows binding of Nef to MHC-I. The endocytosis of MHC-I is aided through the binding of phosphofurin acidic cluster sorting protein (PACS1). MHC-I is transported to the *trans*-Golgi Network where degradation of MHC and dissociation of Nef occur. (B) Coordinated modification of Gag followed by Gag–Gag and other cellular interactions leads to viral assembly and budding. Gag is myristoylated, which targets it to the cellular membrane. Once on the membrane, Gag interacts with other Gag molecules and begins to form the viral core. As this happens, Gag recruits viral RNA and the cellular genes cyclophilin A (CyPA) and Escrt1, which facilitate budding. (C) HIV-1 transcription is aided by Tat's ability to recruit cyclin T1/CDK9 [known as phosphorylated positive transcription elongation factor b (P-TEFb)], which in turn phosphorylates the C-terminal domain of RNA polymerase II (RNA Pol II). In the absence of Tat, transcription pauses between 50 and 100 nucleotides after transcription starts. In the presence of Tat, the TAR element recruits HIV-1 TAT, which recruits cyclin T1/CDK9, this in turn phosphorylates Pol II and leads to activated transcription.



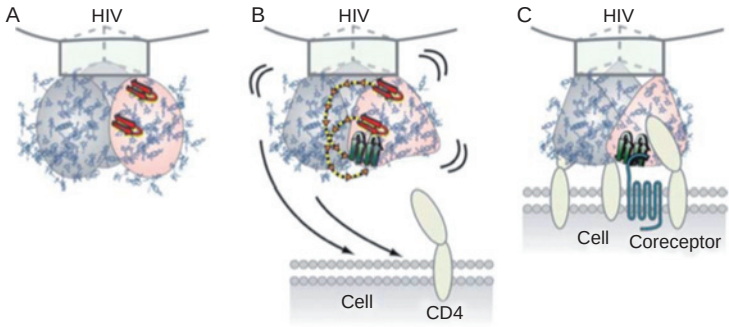
CHAPTER 12, FIGURE 3 Histopathology and SIV-specific *in situ* hybridization of multinucleated giant cells in the lung (A) and choroid plexus of the brain (B) of a RP showing that these cells express SIV viral RNA.



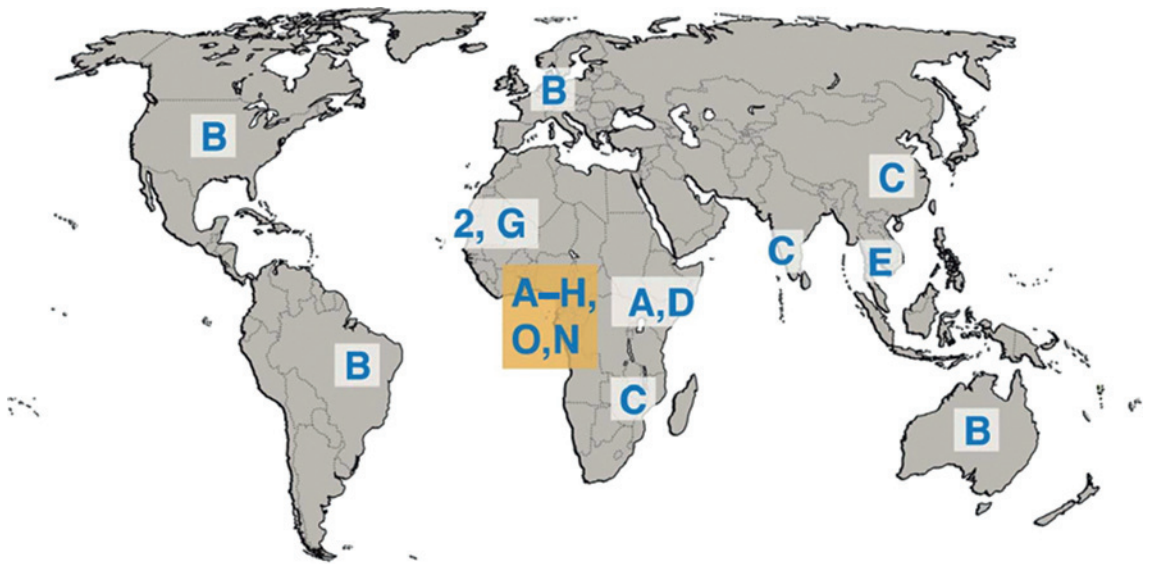
CHAPTER 12, FIGURE 4 Confocal microscopy to identify the target cells of SIV in lesions in the brain of RP macaques. Red indicates HAM56, a marker for macrophages and green indicates SIV RNA. SIV-expressing cells coexpress HAM56, indicating that they are macrophages.



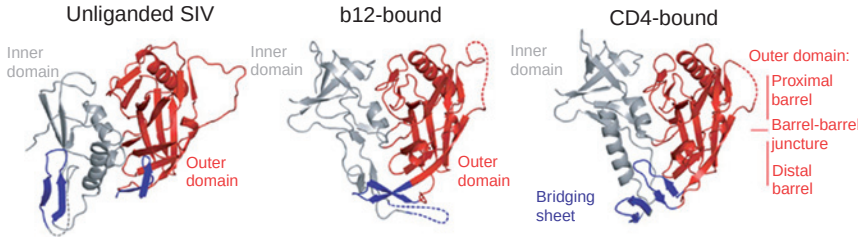
CHAPTER 14, FIGURE 1 (A) Schematic representation of the viral genome containing replication genes (Pol, Vif, Nef, Tat, Rev, Vpu, Vpr) and assembly genes (*gag*, *env*); (B) HIV-1 physical structure. The glycosylated envelope surrounds the viral matrix that contains the viral genome and enzymes required for replication and assembly of the viral particle.



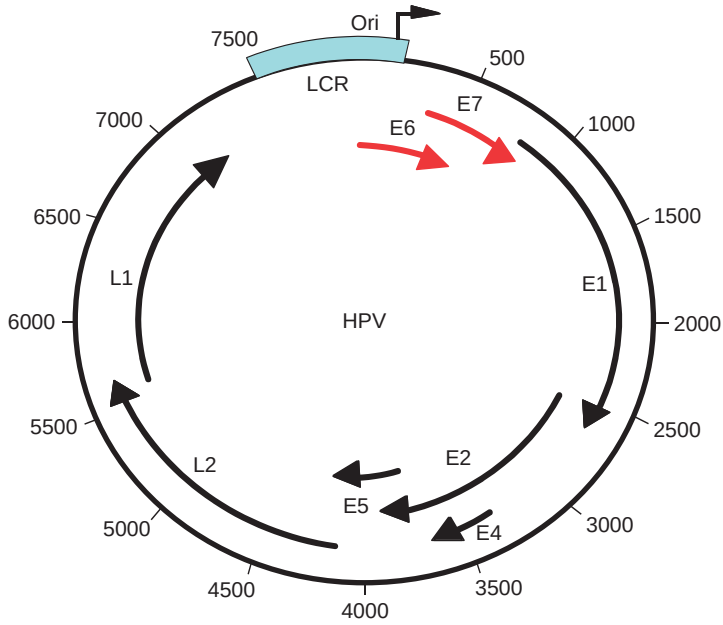
CHAPTER 14, FIGURE 2 Changes in the structure of the HIV envelope on binding to target cells. (A) The heavily glycosylated envelope comprises gp120 and gp41 as a trimer. This structure was solved by Chen *et al.* (2005) for SIV. (B) When the envelope binds to CD4, the gp120 undergoes a conformational change that forms and exposes the binding site for the coreceptor CCR5 or CXCR4 (shown as red β sheet changing to green). (C) This event is followed by conformational changes in gp41 that trigger membranes fusion and virus cell entry (Kwong, 2005). Reprinted by permission from Macmillan Publishers Ltd.: Nature, Kwong 2005, ©2005 Nature Publishing Group.



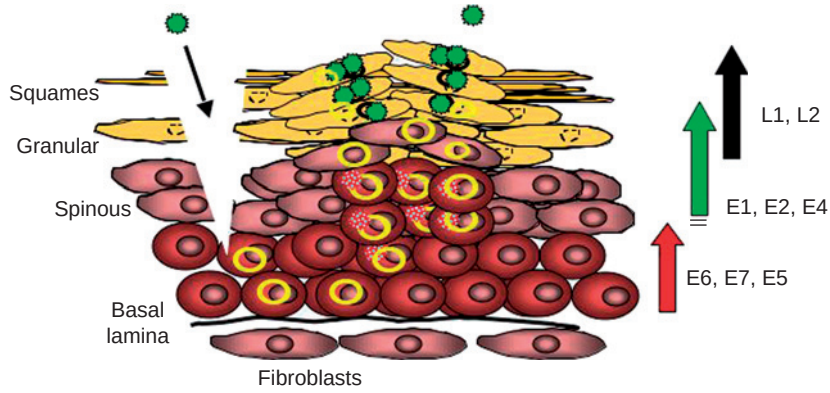
CHAPTER 14, FIGURE 4 Worldwide distribution of the major HIV-1 subtypes (clades) and HIV-2. The subtypes A–H are subdivisions of the M strain; the O and N strains are very close to SIV strains.



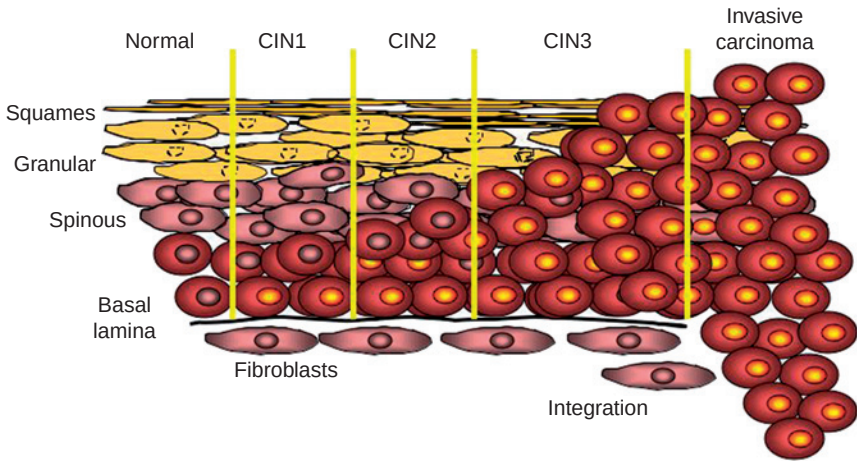
CHAPTER 14, FIGURE 5 Conformational states of gp120. b12- and CD4-bound conformations of gp120 are depicted as in the unliganded conformation. Inner domains are gray, outer domains are red, with the disordered regions as dashed lines. Inner domains are depicted in red and the bridging sheet is shown in blue. Reprinted by permission from Macmillan Publishers Ltd.: Nature, Zhou *et al*., 2007, ©2007 Nature Publishing Group.



CHAPTER 16, FIGURE 1 The HPV genome is a 7.9kb double-stranded circular genome. The genome is controlled by a single keratin-dependent promoter element; the long control region (LCR; *in blue*). At the 3′ end of the LCR is the origin of replication (*nucleotide position 1*). E6, E7 (*red*), and E5 are viral oncogenes; E1 and E2 early genes encode replication proteins. The E4 ORF is actually expressed early and late in the viral life cycle. The late genes, L1 and L2, are the major and minor capsid genes, respectively. The viral protein functions are detailed in Table I.



CHAPTER 16, FIGURE 2 The HPV life cycle. Virions enter the stratified epithelium through a site of wounding, where they gain access to the mitotically active basal-layer keratinocytes. During the maintenance phase, expression of E6, E7, and E5 induces cell proliferation, and the viral genome is replicated extrachromosomally at low-copy number (5–50 copies per cell). As the cells differentiate, the expression level of E1, E2, and E4 increases in the spinous layer. A transition from theta to rolling-circle replication results in an increase in copy number up to 100–1000 copies per cell. Postamplification, high levels of L1 and L2 capsid genes are expressed and capsid assembly occurs in the granular and squamous layers of the stratified epithelium. Progeny virus is released by desquamation.



CHAPTER 16, FIGURE 3 Progression from a benign cervical lesion to invasive cervical cancer. In the diagram, HPV-positive cells are depicted by yellow nuclei. Infection by oncogenic HPV types, especially HPV16, can cause formation of a benign wart, low or high-grade dysplasia. CIN 1 and CIN 2 designations are reversible forms of precancerous lesions and CIN 3 is irreversible. Carcinoma *in situ* occurs many years after an infection. This results from the effects of HPV genes, particularly those encoding E6 and E7, which are the two viral oncoproteins that are preferentially retained and expressed in cervical cancers by integration of the viral DNA into the host genome.