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## Preface

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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.

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

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## **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



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.

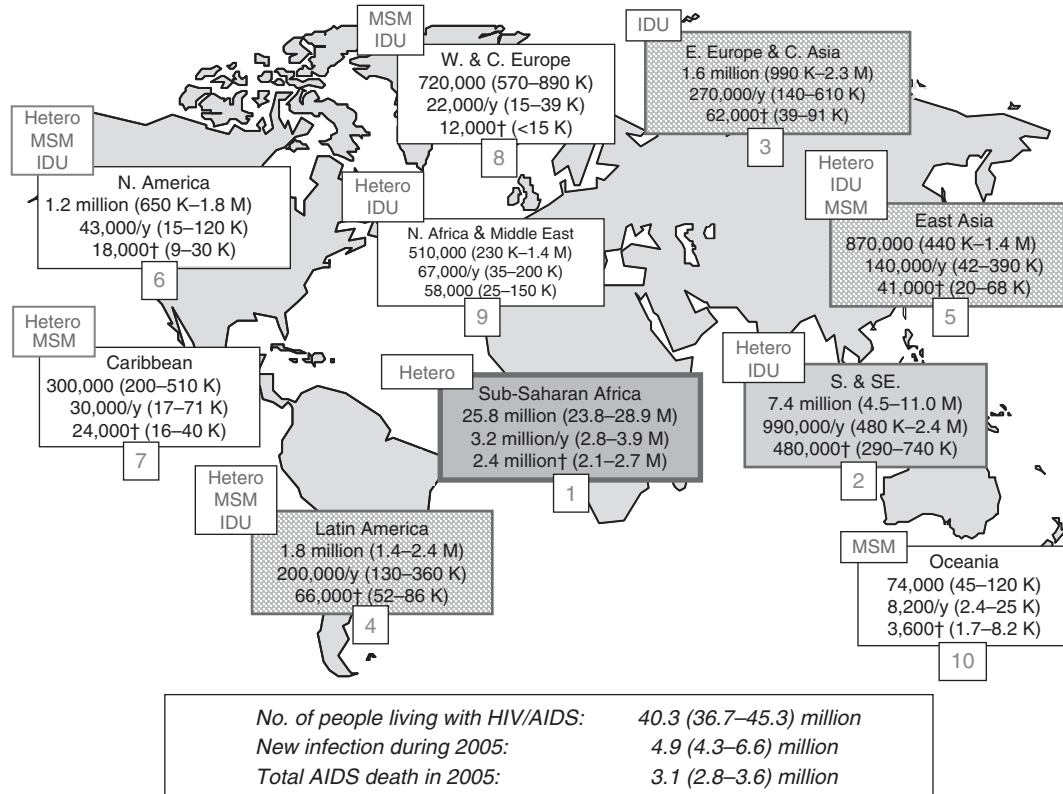
## II. Introduction

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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)

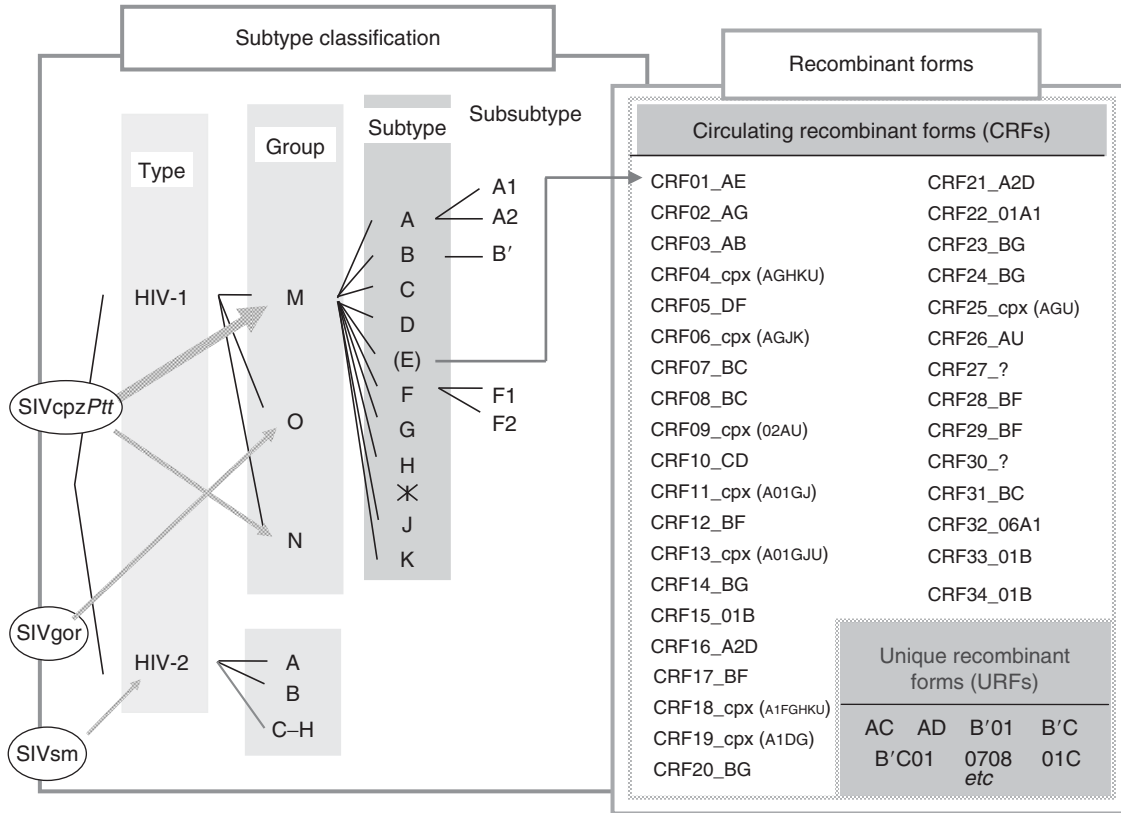
Etiologic agents for AIDS are subdivided into two related human retroviruses (lentiviruses): HIV-1 (HIV type 1) and HIV-2 (HIV type 2). HIV-1 is distributed worldwide, accounting for the majority of HIV infections. By contrast, HIV-2 is confined to West Africa and southern/western India (Schim van der 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



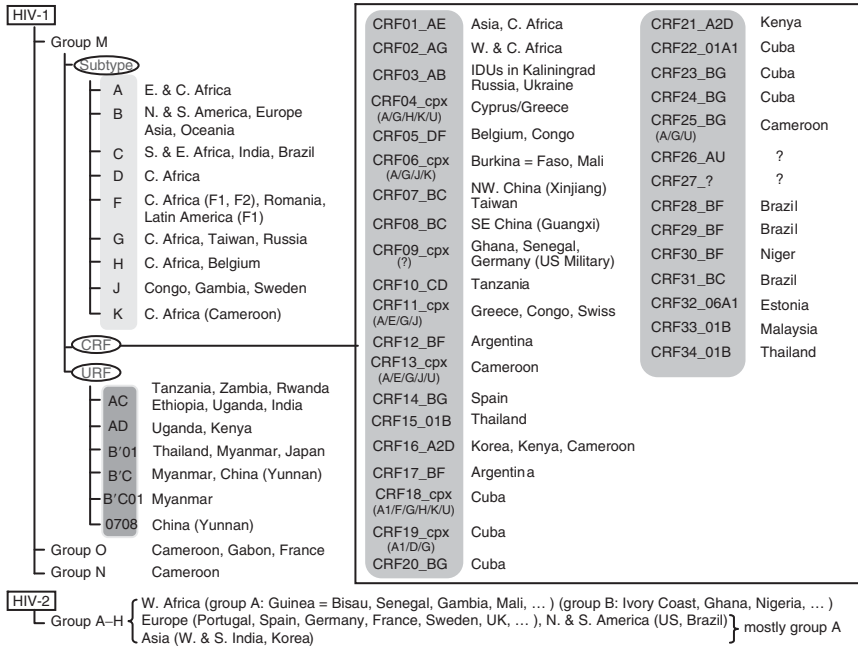
**FIGURE 2** Classification of HIV genotypes and their origins. HIVs are classified based on the following four different strata: types (types 1 and 2), groups (M, O, and N for HIV-1; A–G for HIV-2), subtypes (A–K for HIV-1), and subsubtypes (A1 and A2, F1 and F2). HIV-1 recombinants in pandemic strains 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). Intra-patient 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

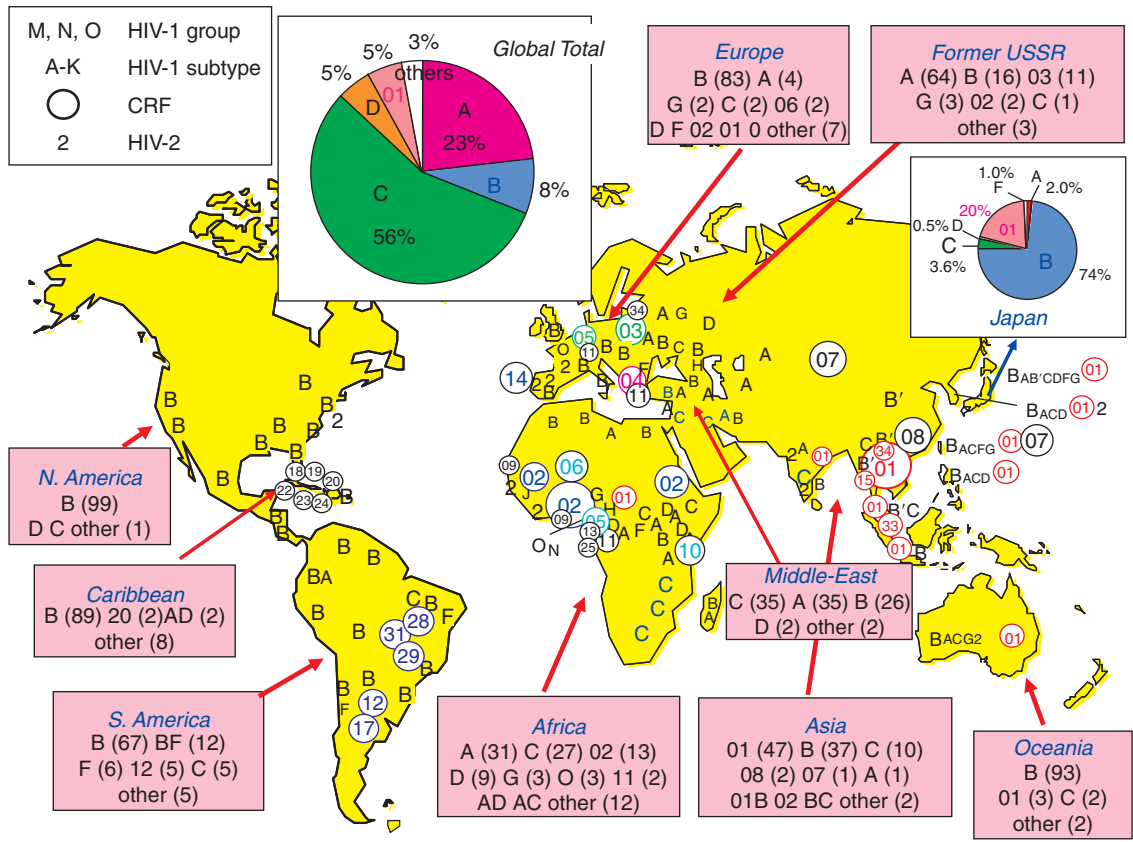


**FIGURE 3** Geographical distribution of HIV genotypes. The global distributions of HIV-1 genotypes (groups, subtypes/subsubtypes, and CRFs/URFs) and HIV-2 groups are shown.

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/](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

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### 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 Bhamarapavati, 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, Malaysia, 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 (Tovanabuttra *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/](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

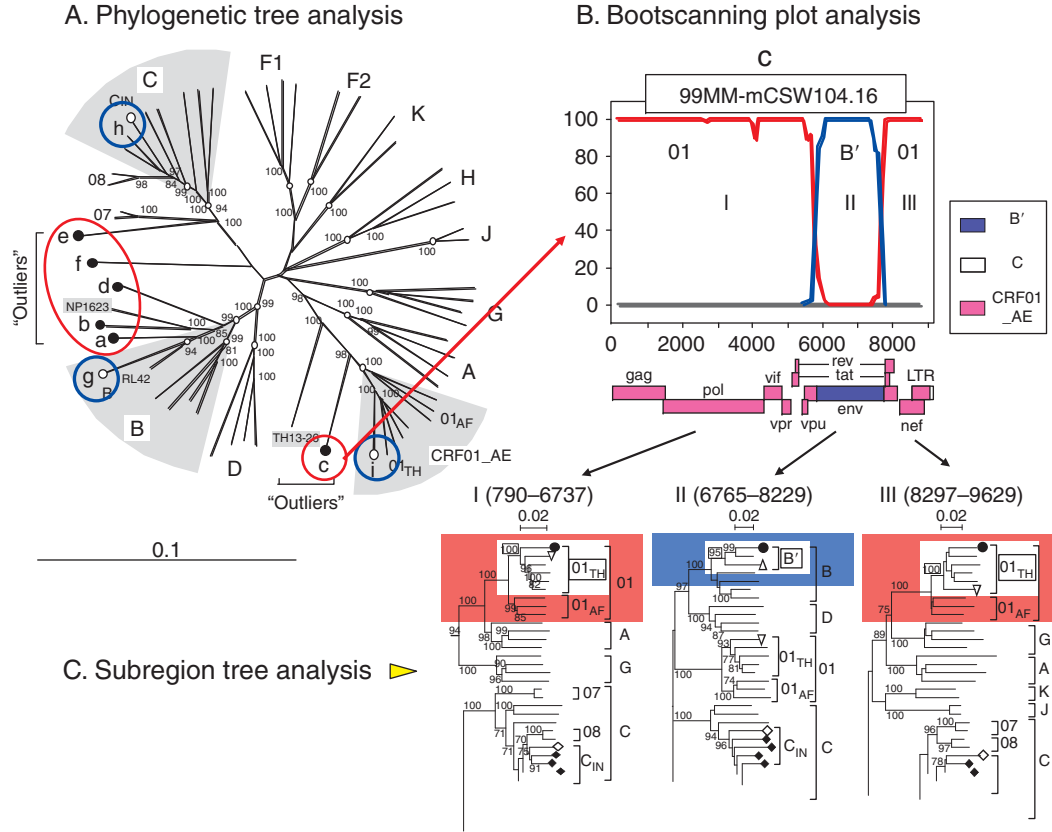
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### A. Phylogenetic Sequence Analysis

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

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(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-cpzPtt 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-cpzPtt 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

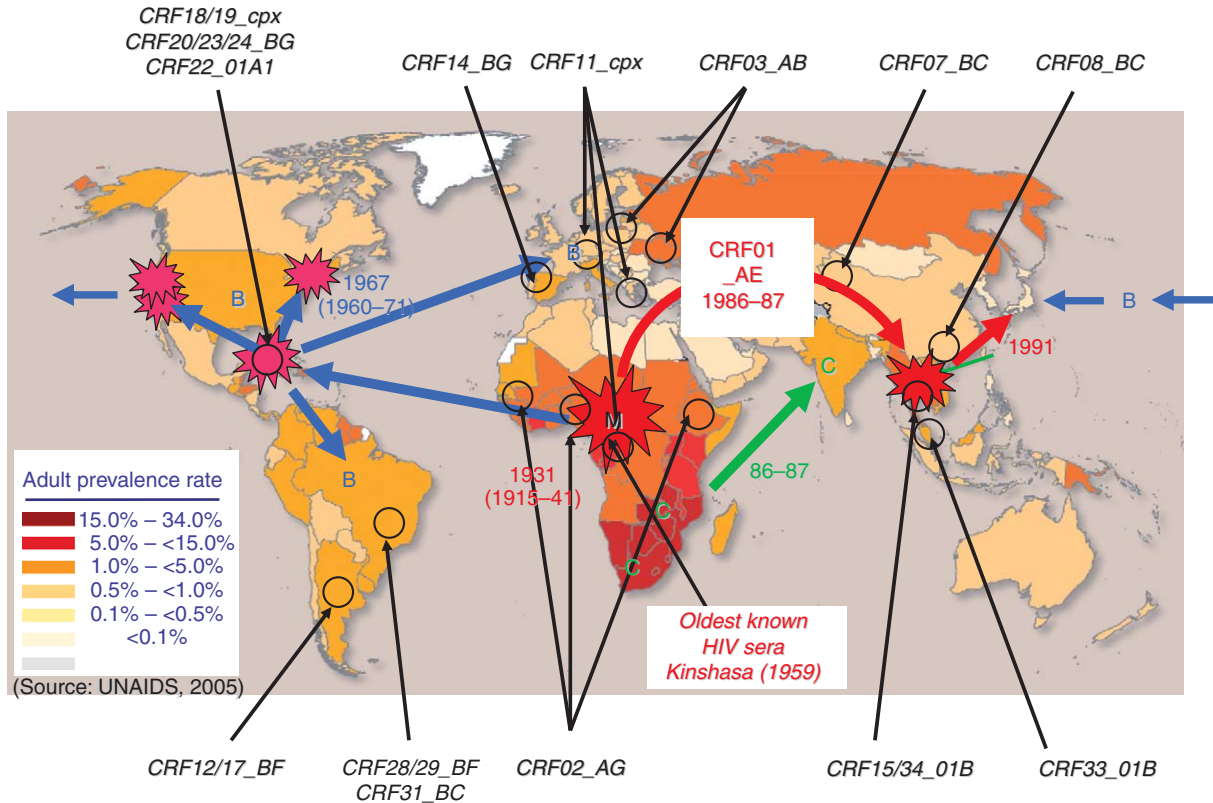
Korber *et al.* (2000) estimated the date of the most recent common ancestor (MRCA) of HIV-1 group M to be 1931 [95% confidence interval (CI): 1915–1941], suggesting that HIV-1 group M began its expansion in human population roughly 70 years ago. The phylogenetic analyses assuming molecular clock suggested 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

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### A. HIV-1 Subtypes and Disease Progression

It has been suggested that HIV-1 subtypes could influence viral transmissibility and pathogenicity. 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- $\alpha$ ) is increased in subtype C long terminal repeat (LTR) with nuclear factor  $\kappa$ B (NF $\kappa$ B) 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 either 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 have 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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

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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 pathogenicity 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.

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# Current Clinical Treatments of AIDS

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## 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

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

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### A. Nucleoside/Nucleotide Reverse Transcriptase Inhibitors

#### I. 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- $\gamma$ , 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- $\gamma$  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- $\beta$ -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<sup>®</sup>) or with both 3TC and ABC (Trizivir<sup>®</sup>). 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- $\alpha$ , 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

<i>Antiretroviral</i>			
<i>Daily Dose</i>		<i>Dosing in renal insufficiency</i>	
Abacavir*		No need for dosage adjustment	
300 mg PO BID			
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<sup>®</sup>, Combivir<sup>®</sup>, Trizivir<sup>®</sup>, Epzicom<sup>®</sup>—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 (Podzamczar *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<sup>®</sup>) and ZDV plus ABC (Trizivir<sup>®</sup>).

*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<sup>®</sup>) and with both tenofovir and efavirenz (Atripla<sup>®</sup>). Of note, Atripla<sup>®</sup> 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<sup>®</sup>) (Sosa *et al.*, 2005). ABC is also a component for the three-NRTI formulation with ZDV and 3TC (Trizivir<sup>®</sup>).

*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 (C<sub>max</sub> 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<sup>®</sup>). 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 coinfecting patient abruptly discontinues TDF.

*Drug Interactions* TDF is not metabolized by the cytochrome P450 system, nor does it exert any effects 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**

### **I. 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 (C<sub>min</sub>). Patients able to consistently maintain the C<sub>min</sub> 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<sup>3</sup> and men with a pretreatment CD4 >400 cells/mm<sup>3</sup> 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 sub-therapeutic 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™).

**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<sub>2</sub> 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 with 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 mg 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 ( $2 \times 500$ -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 (C<sub>max</sub>) 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 of 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 C<sub>min</sub> 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-naïve 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-naïve 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-naïve 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 major inhibitor 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<sub>2</sub> 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 C<sub>min</sub> 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 C<sub>min</sub> were decreased by  $\sim 25\%$  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 elevated dramatically (100% and 55%, respectively) (Baldwin *et al.*, 1998). Serum half-life is optimal ( $\sim 6$  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 hepatotoxicity and intracranial hemorrhage, both warranting a black box warning. In clinical trials, TPV-treated patients were 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 than 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 C<sub>max</sub> ([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 C<sub>min</sub> 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 C<sub>max</sub> (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 box 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

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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).

### I. 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<sup>®</sup>) 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<sup>®</sup> 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<sup>3</sup> in the tenofovir/FTC arm and 237 cells/mm<sup>3</sup> 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<sup>®</sup>) 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-naïve 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<sup>3</sup> and men with a count  $>400$  cells/mm<sup>3</sup> 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<sup>®</sup>, 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-naïve 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 than to hold all HAART.

#### IV. Conclusion

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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.

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# HIV-1-Specific Immune Response

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## I. Introduction

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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.



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

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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

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#### 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- $\gamma$  (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**

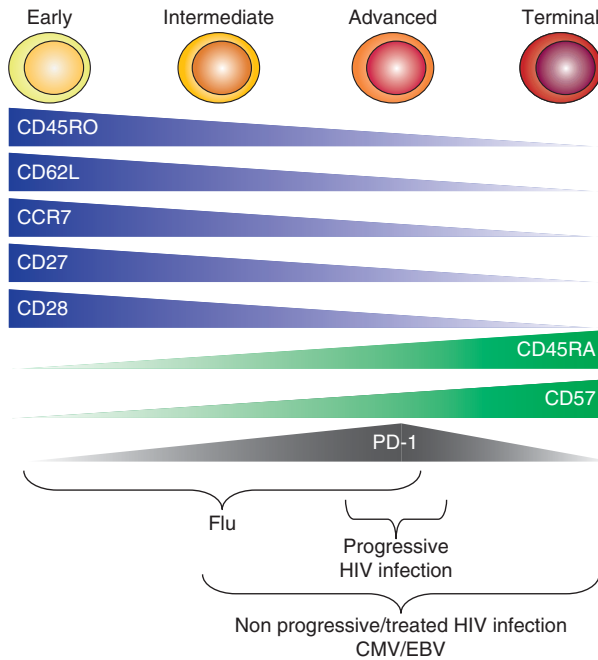
The characterization of the HIV-1-specific T-cell responses has been the objective of a very large number of studies and any type of phenotypic and functional abnormalities have been described (Appay *et al.*, 2002; Autran *et al.*, 1995, Brenchley *et al.*, 2003; Champagne *et al.*, 2001; Gillespie *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 (Kaeche *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-7R $\alpha$ , 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).

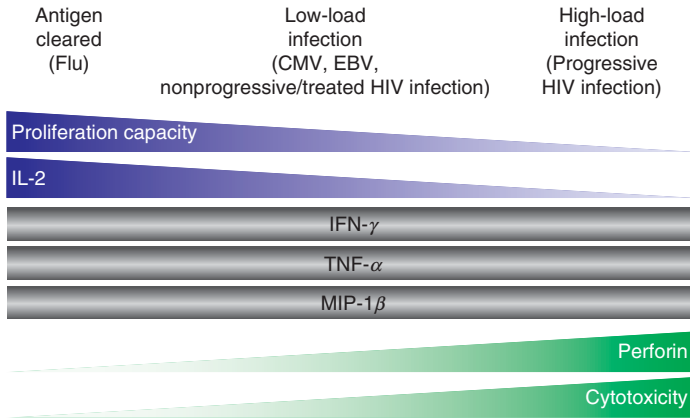
Therefore, there is a good correlation between the phenotypic profiles of both virus-specific CD4 and CD8 T cells and the control of virus replication. In this regard, it is worth to mention that a number of studies have shown a correlation between the magnitude of the HIV-1-specific  $T_{ET}$  CD8 T-cell 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- $\gamma$ , TNF- $\alpha$ , and MIP-1 $\beta$ , 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- $\gamma$ , three functionally distinct populations of antigen-specific CD4 T cells (single IL-2, dual IL-2/IFN- $\gamma$ , and single IFN- $\gamma$ ) 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- $\gamma$  antigen-specific CD4 T-cell populations have intrinsic proliferation capacity while single IFN- $\gamma$  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- $\alpha$  and, based on the secretion of IFN- $\gamma$ , two equally represented cell populations of single IFN- $\gamma$  and dual IFN- $\gamma$ /TNF- $\alpha$  can be identified.

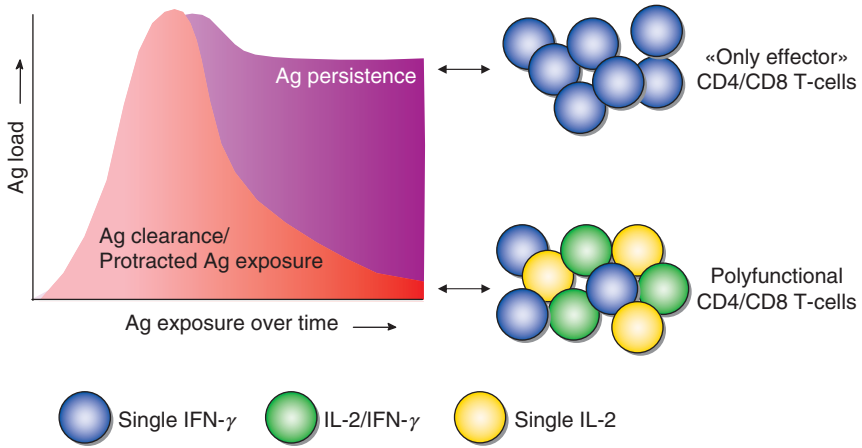
With regard to CD8 T cells, two cell populations of antigen-specific CD8 T cells (dual IL-2/IFN- $\gamma$  and single IFN- $\gamma$ ) can be defined based on the ability to secrete IL-2 and IFN- $\gamma$  (Zimmerli *et al.*, 2005). Both CD8 T-cell populations are cytotoxic as measured by the expression 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- $\gamma$  compared with dual IL-2/IFN- $\gamma$  CD8 T cells. The dual IL-2/IFN- $\gamma$  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- $\gamma$  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- $\alpha$  secreting cells were also IFN- $\gamma$  secreting cells, that is, dual IFN- $\gamma$ /TNF- $\alpha$ . Finally, the assessment of MIP-1 $\beta$  has been proposed to define a variable percentage (up to 30%) of antigen-specific CD8 T cells not producing IL-2, TNF- $\alpha$ , and IFN- $\gamma$  (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- $\gamma$ , TNF- $\alpha$ , MIP-1 $\beta$ , 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- $\gamma$ , TNF- $\alpha$ , and MIP-1 $\beta$ , 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- $\gamma$  and TNF- $\alpha$  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- $\gamma$  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- $\gamma$  and TNF- $\alpha$  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- $\gamma$  and dual IFN- $\gamma$ /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.

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# Targeting HIV Attachment and Entry for Therapy

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## 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

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

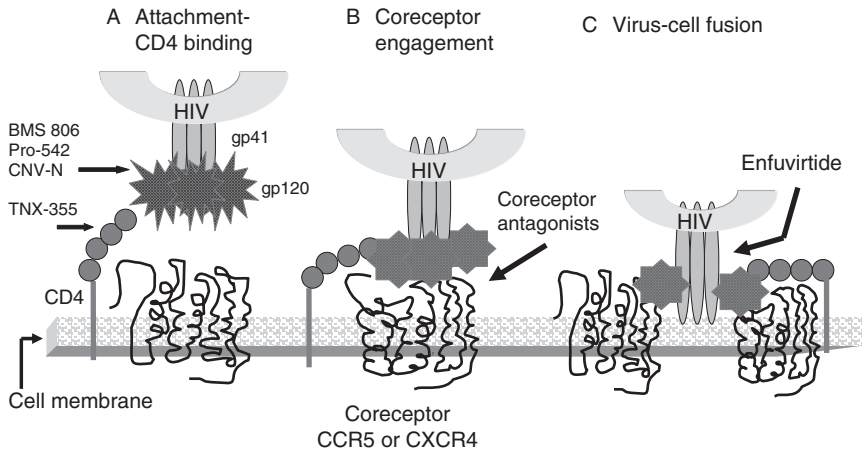
## II. Background

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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 I** 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 gp41 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 ( $K_d \sim 30\text{--}35$  nM) to gp120 proteins from clade B viruses. However, RC-101 binding to nonclade B envelopes was substantially reduced ( $K_d \sim 200\text{--}700$  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

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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-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ ), 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

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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-1 $\alpha$ , MIP-1 $\beta$ , 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.

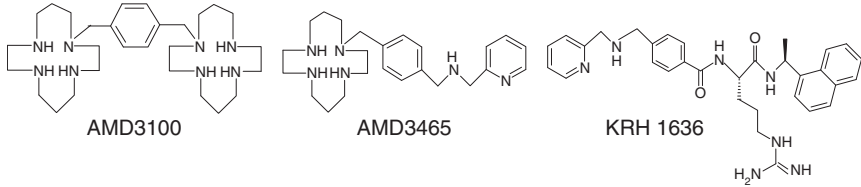
### **1. Peptide Inhibitors of CXCR4**

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.

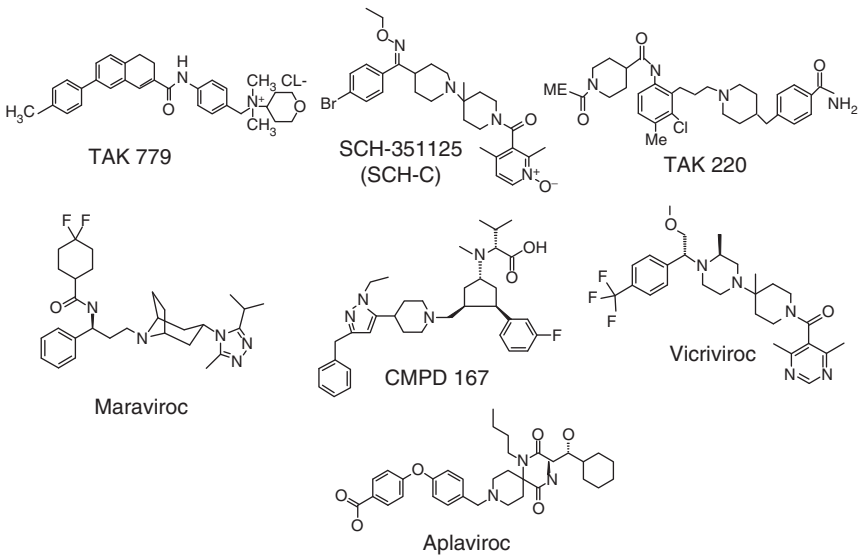
### **2. Small Molecule CXCR4 Inhibitors**

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 molecule 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<sub>10</sub> following AMD3100 dosing, providing proof-of-concept for this

## A CXCR4 antagonists



## B CCR5 antagonists



**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<sub>50</sub> 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 (Schlyer 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 potentially 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 resulting in a dose-dependent decrease in viral load up to  $\sim 1$  log<sub>10</sub> 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

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

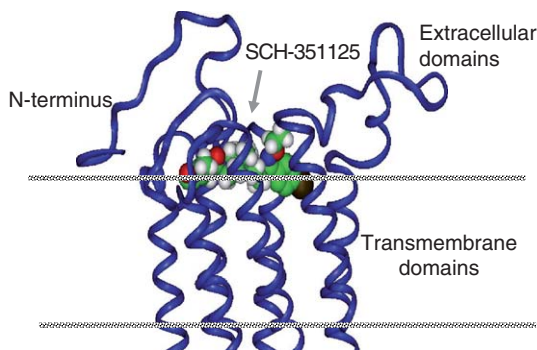
Fusion	CXCR4	AMD 3100	Small molecule	Stabilize conformation of CXCR4 not recognized by gp120
		AMD 070	Small molecule	
		KRH 1636	Small molecule	
	gp41	KRH 3140	Small molecule	Inhibition of gp120-CXCR4 binding
		ALX40-C	9-amino acid peptide	
		T-22	18-amino acid peptide	
		T-140	14-amino acid peptide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

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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 conformation 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

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### 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 gp41 conformational change (Frey *et al.*, 2006). These compounds are believed to bind to the gp41 inner core and prevent viral fusion with IC<sub>50</sub> 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

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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<sub>50</sub> 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<sub>50</sub> 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

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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.

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# Inhibitors of HIV-1 Reverse Transcriptase

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## I. Chapter Overview

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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

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

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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)

<i>Brand name</i>	<i>Generic name</i>	<i>Manufacturer</i>
<i>Nucleoside reverse transcriptase inhibitors (NRTIs)</i>		
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
<i>Nucleotide reverse transcriptase inhibitors (NtRTIs)</i>		
Viread	Tenofovir disoproxil fumarate (TDF)	Gilead
<i>Nonnucleoside reverse transcriptase inhibitors (NNRTIs)</i>		
Rescriptor	Delavirdine (DLV)	Pfizer
Sustiva	Efavirenz (EFV)	Bristol Myers-Squibb
Viramune	Nevirapine (NVP)	Boehringer-Ingelheim
<i>Single dose combination products</i>		
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

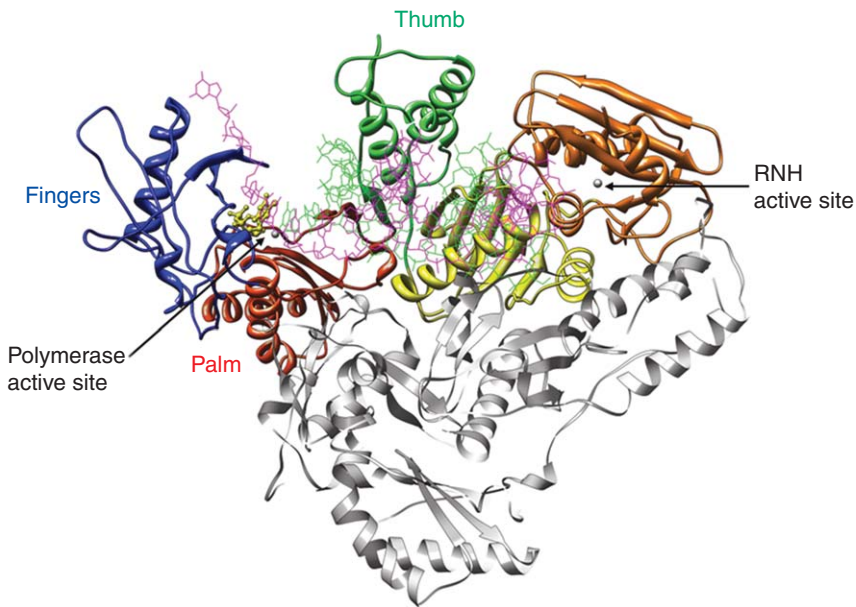
The conversion of retroviral genomic RNA into double-stranded DNA is complex, yet all steps in this conversion are catalyzed by the retroviral RT. Accordingly, RT must be a multifunctional enzyme. RT has two types of DNA polymerase activity. The RNA-dependent DNA polymerase (RDDP) activity of RT prepares 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

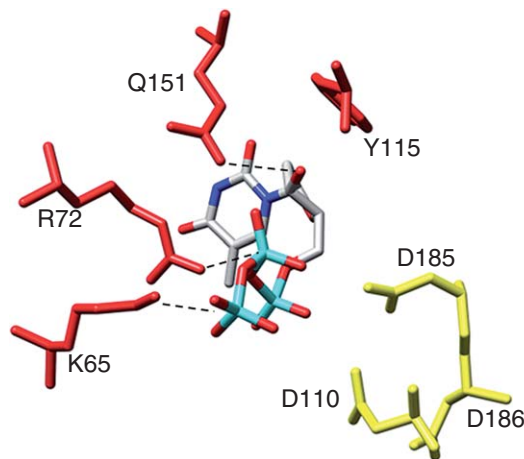
The gene for HIV-1 RT encodes a 66-kDa protein. However, HIV-1 RT is an obligatory heterodimer consisting of two subunits of 66 and 51 kDa (Fig. 1). The p51 subunit is derived from p66 former by HIV-1 protease-catalyzed removal of the 15-kDa C-terminal RNH domain during HIV-1 assembly and maturation (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<sup>Lys3</sup> 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  $\alpha$ -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  $\alpha$ -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<sub>n+1</sub>-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

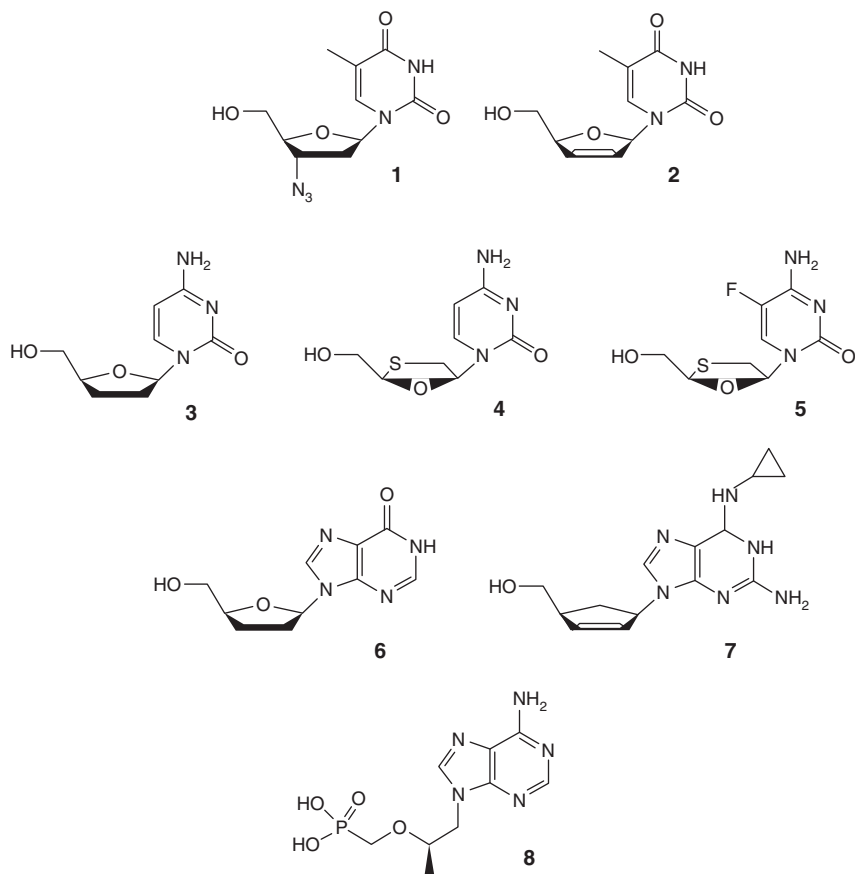
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NRTIs are analogs of naturally occurring deoxyribonucleosides, but lack the 3'-hydroxyl group (Fig. 3). NRTI have been used clinically in the treatment of HIV-1 infection since 1986, and one nucleotide and seven NRTIs are in current clinical use (Table 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'-hydroxyl 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 bioavailability 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; (-)- $\beta$ -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 (Soudeyys *et al.*, 1991). The anti-HIV inhibitory concentration (IC<sub>50</sub>) values for 3TC in different cell types range from 4 nM to 0.67  $\mu$ M (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; (-)- $\beta$ -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).



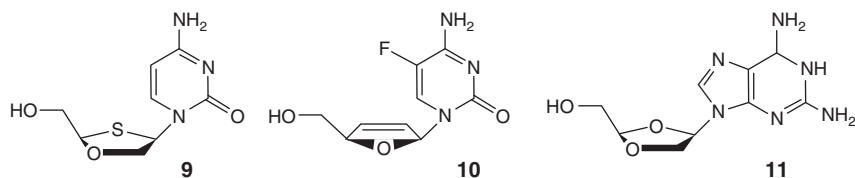
## 8. Viread (Tenofovir; 9-[2-(Phosphonomethoxy) Propyl]Adenine; TDF)

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-isopropoxycarbonyloxymethyl 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 coinfecting 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- $\beta$ -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 [( $-$ )- $\beta$ -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 ( $-$ )- $\beta$ -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  $\beta$ -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'-dideoxy-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 pyrophosphate (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).

### **I. 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.

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

**TABLE II** Clinically Observed Mutations in Reverse Transcriptase Associated with HIV Resistance to Individual NRTI

<i>NRTI</i>	<i>Mutations</i>	<i>Resistance phenotype</i>
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 <sup>a</sup>
Abacavir	K65R, L74V, Y115F, M184V	Discrimination, phosphorolysis
Lamivudine (3TC)	E44D, M184V	Discrimination <sup>a</sup>
Tenofovir (TDF)	K65R	Discrimination, phosphorolysis
Emtricitabine (FTC)	M184V, K65R (?)	Discrimination (?) <sup>a</sup>

<sup>a</sup>NRTI based on cytidine appear to be quite refractory to the phosphorolytic excision phenotype.

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  $\beta$ -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  $\beta$ -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  $\sim$ 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  $\gamma$ -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.

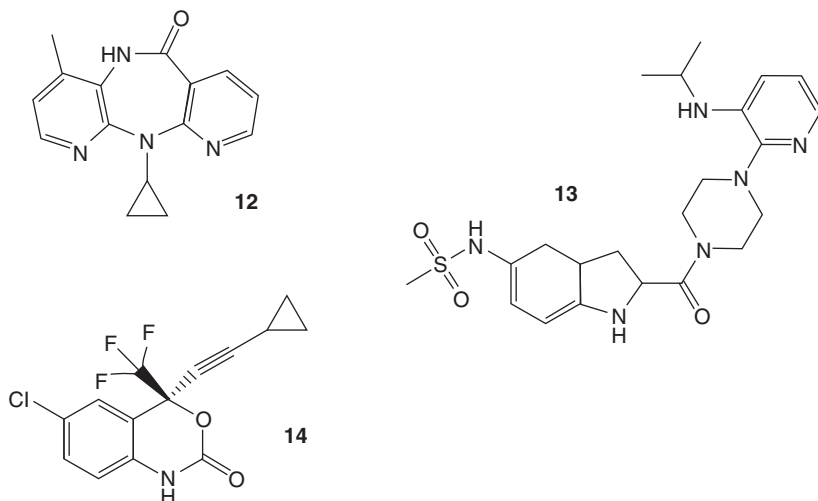
## V. Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)

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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).

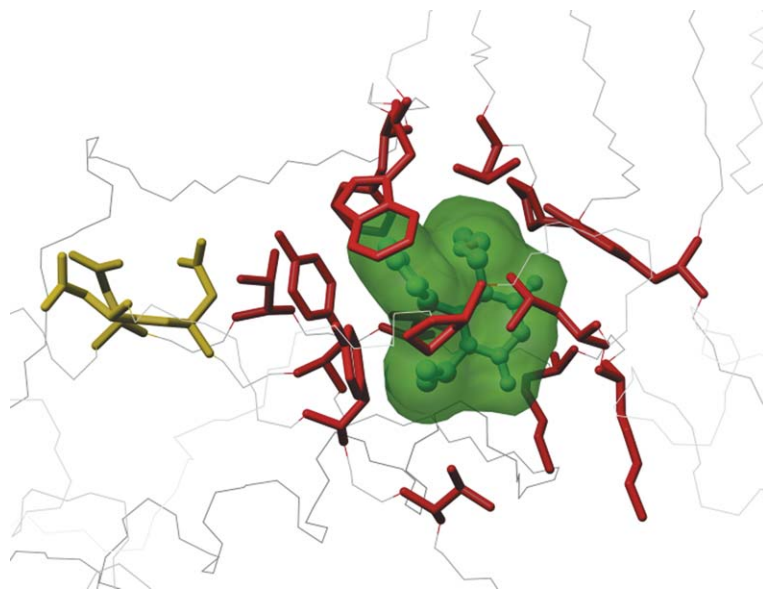
### A. Mechanisms of NNRTI Inhibition

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  $\pi$ -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 manner, closing down around the surface of the drug to make tight van der Waals contacts (Kroeger-Smith *et al.*, 1995).



**FIGURE 5** Structures of nonnucleoside RT inhibitors (NNRTIs) approved for clinical use. 12, Nevirapine (NVP); 13, delavirdine (DLV); 14, efavirenz (EFV).

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 may be important for T/P translocation during DNA polymerization (Kohlstaedt *et al.*, 1992; Tantillo *et al.*, 1994). Binding of NNRTIs may



**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).

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 ( $\approx 40\%$ ). 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.

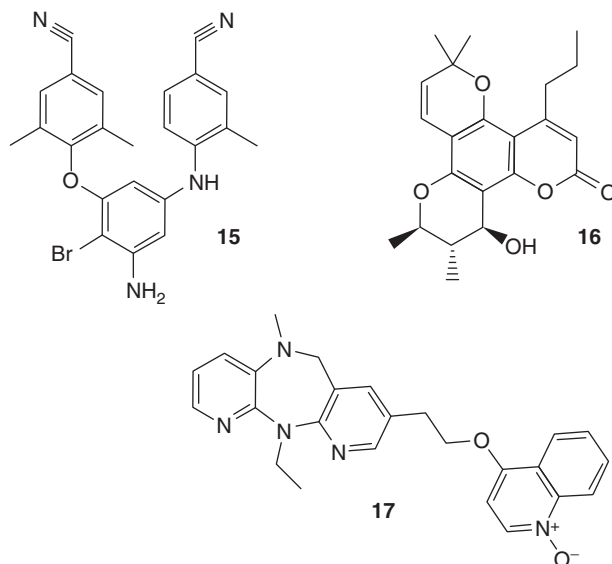
Despite these concerns, EFV has become the “mainstay” of NNRTIs, mainly because of excellent activity against many NNRTI-resistant HIV-1 strains. A preferred first-line therapy is the combination of EFV with the NRTIs AZT plus 3TC, or TDF plus FTC. The single dose combination, EFV + TDF + FTC, is marketed 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 ( $EC_{50} = 3.5$   $\mu$ M). 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 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



**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.

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 dipyrano-coumarin 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  $\pi$ -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 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.

#### I. 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

**TABLE III** Clinically Significant Mutations in Reverse Transcriptase Associated with HIV Resistance to NNRTI

<i>Drug</i>	<i>Mutations</i>	<i>Resistance phenotype</i>
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

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 (Bachelier *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  $\pi$ -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  $\pi$ -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).

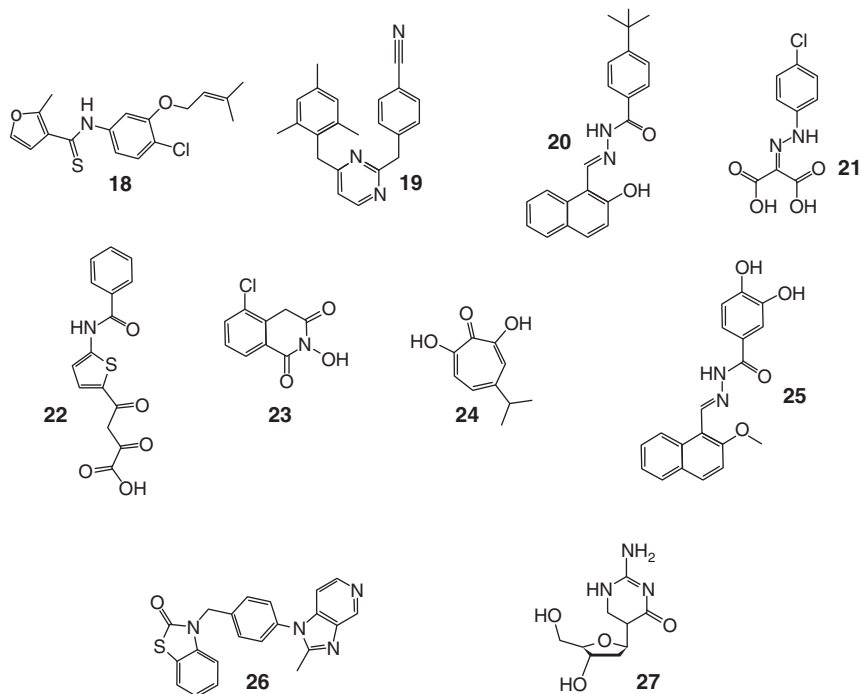
## E. Interactions Between NNRTI and NRTI Resistance Mutations

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 activation, 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



**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.

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

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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

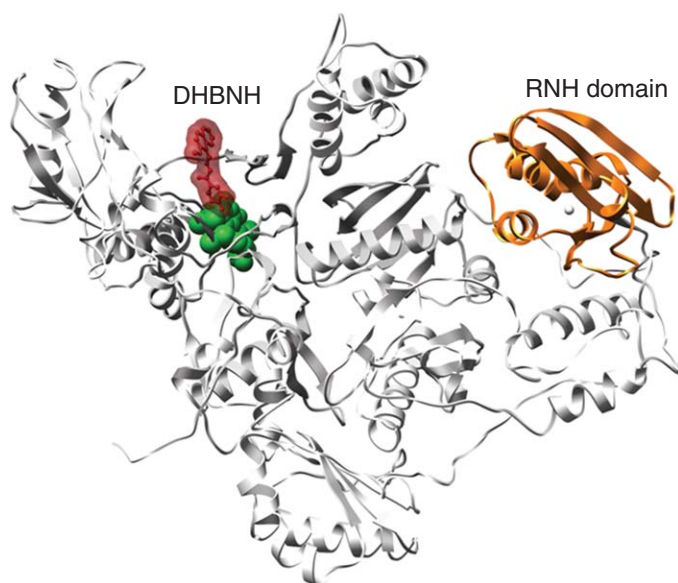
complete viral dsDNA synthesis. The removal of the RNA in the RNA/DNA heteroduplex is carried out by the RT-associated enzyme activity, RNH. The RT DNA polymerase and RNH active sites are spatially distinct, with RNH activity localized in the C-terminal 15-kDa subdomain of the 66-kDa subunit of the HIV-1 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 (Budihias *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 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



**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).

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)- $\beta$ -D-ribofuranosyl]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)-3-ethylthymine (TSAOe<sup>3</sup>T) is a highly modified nucleoside that acts in a manner similar to NNRTIs (Balzarini *et al.*, 1992a,b). Interaction of TSAOe<sup>3</sup>T with RT results in a 4 kcal mol<sup>-1</sup> 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

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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.

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

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## I. Chapter Overview

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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.

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.

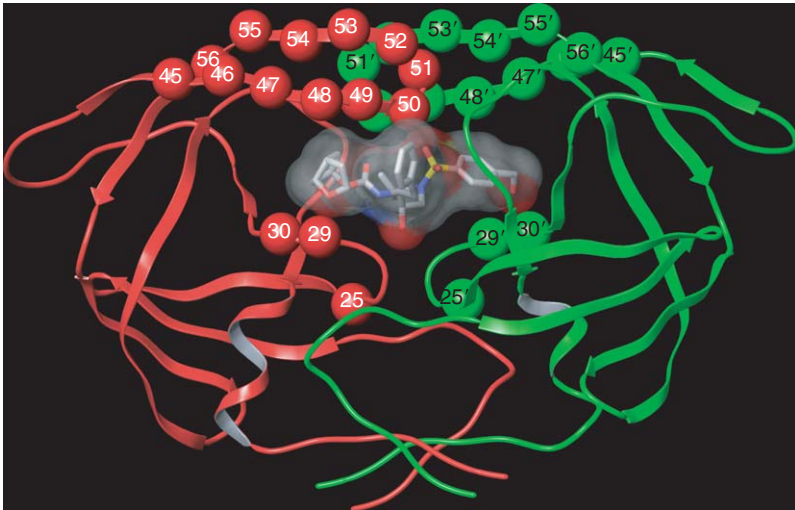
## III. Targeting Viral Protease ---

### 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  $\beta$ -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  $\beta$ -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

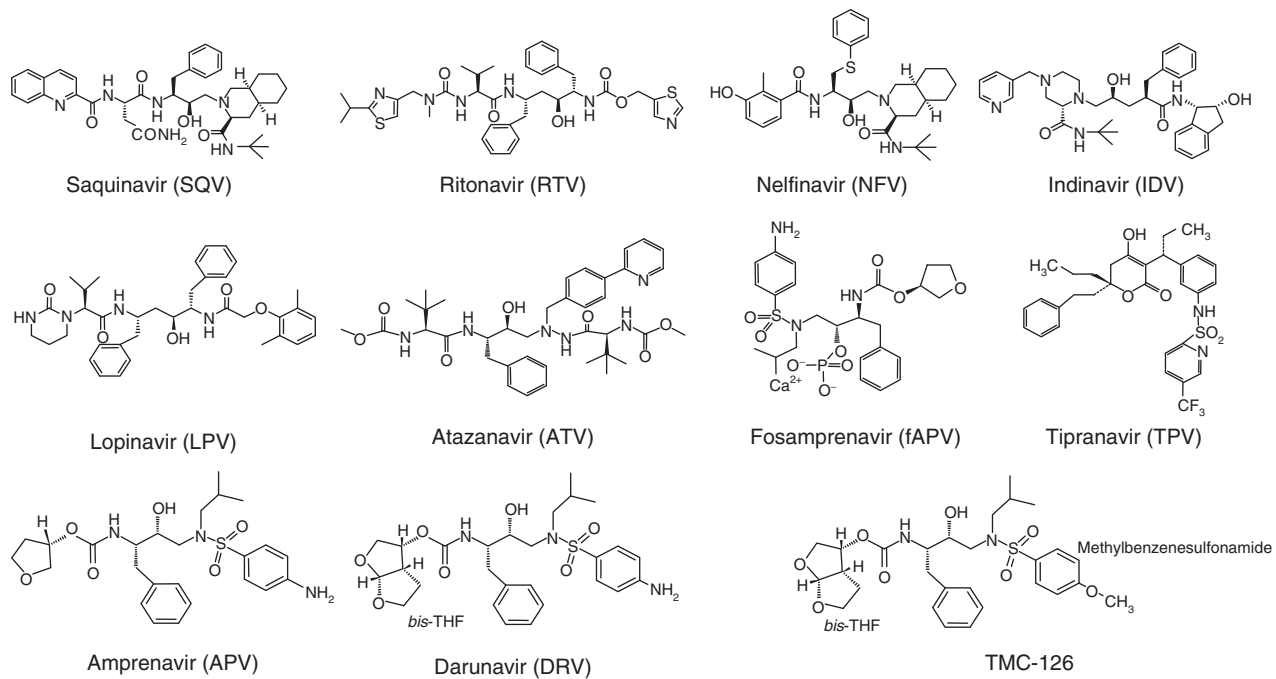
In theory, antiviral drugs exert their effects by interacting with viral structural components, virally encoded enzymes, viral genomes, or specific host proteins such as cellular receptors, enzymes, or other factors required for viral replication (Mitsuya and Broder, 1987; Mitsuya and Erickson, 1999; 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 (C<sub>2</sub>) 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

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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-naïve 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

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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 ( $C_{\text{trough}}$ ), 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 pharmacokinetic 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-naïve 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-naïve 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

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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 Podzamczar, 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 I** Mutations Associated with Resistance to Currently Approved Protease Inhibitors

<i>Mutations conferring high/intermediate resistance</i>							
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 <sup>a</sup>		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 <sup>b</sup>	L33F			I50V		I84V	

<sup>a</sup>Fosamprenavir is the prodrug of amprenavir; the latter is no longer manufactured.

<sup>b</sup>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'- $\beta$ -fluoro-2',3'-dideoxyadenosine (FddA or lodenosine), *in vitro* (HIV-1<sub>FddA</sub><sup>R</sup>), all clones derived from HIV-1<sub>FddA</sub><sup>R</sup> 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<sub>LAI</sub>) 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<sup>+</sup>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.

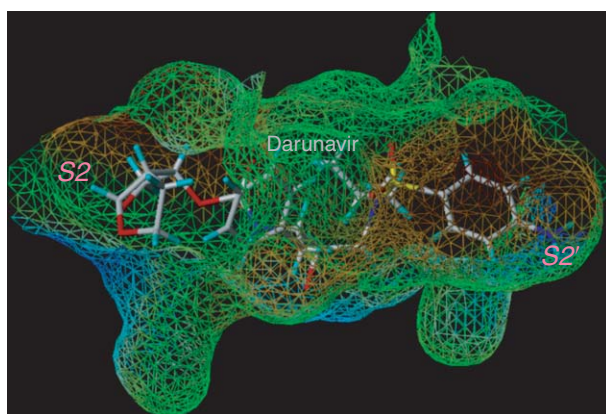
Tamiya *et al.* (2004) have also identified unique insertions (TGNS, SQVN, AQQA, SRPE, APP, and/or PTAPPA) near the p17/p24 and p1/p6 Gag cleavage sites in addition to the known multiple amino acid substitutions within the protease in full-length molecular infectious multidrug-resistant (MDR) HIV-1 clones 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

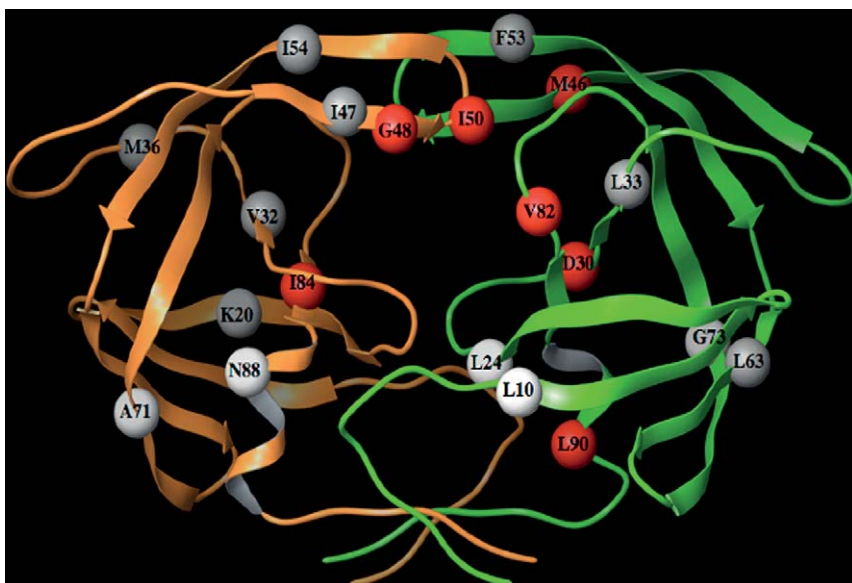
### A. Mutations That Allow Discrimination of PIs from Natural Substrates

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**

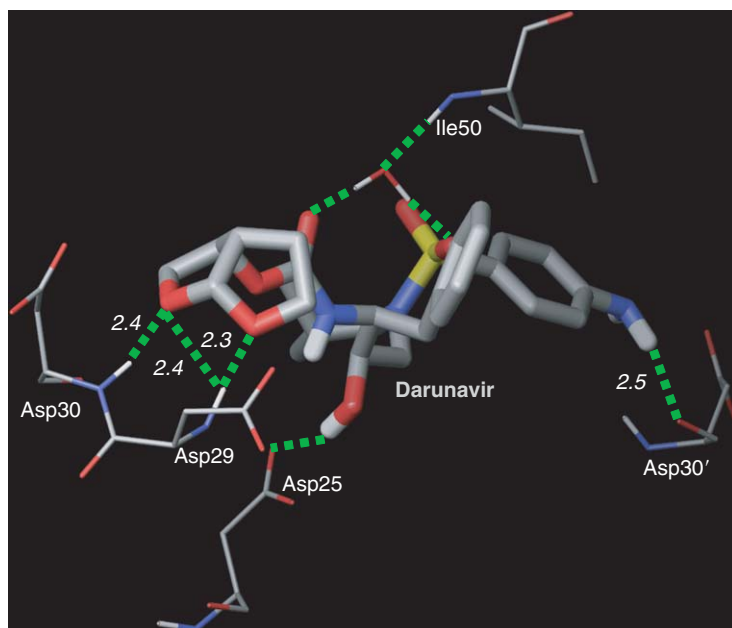
Of the currently approved PIs, APV fits predominantly within the substrate envelope ([King \*et al.\*, 2004b](#)). The mutation profile for APV is also different compared with that for other PIs, providing more evidence that PIs that have greater resemblance to natural substrates will be less affected 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*-tetrahydrofuryl (*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-methoxybenzenesulfonamide.

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-methoxybenzenesulfonamide 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<sub>LAI</sub> 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  $\mu$ M, 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  $\mu$ M DRV), the virus had acquired  $\sim$ 170-fold IC<sub>50</sub> increases than that against HIV-1<sub>NL4-3</sub>. 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  $\mu$ M DRV (HIV-1<sub>DRV-P30</sub>) was titrated and examined for its susceptibility to DRV and other PIs using p24 assay. HIV-1<sub>DRV-P30</sub> was found highly resistant to DRV (110-fold greater IC<sub>50</sub> than that against HIV-1<sub>NL4-3</sub>) (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).

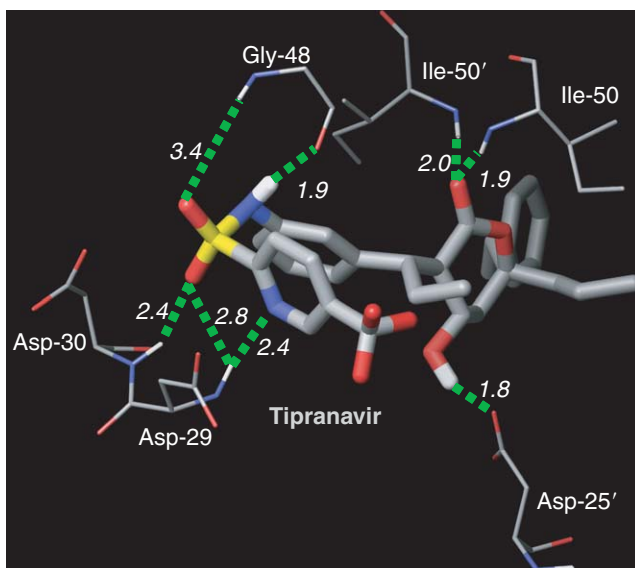
At present, no randomized comparison data of TPV versus DRV are available, although there are a few reports on the benefits of TPV and DRV in those who have failed combination therapy with other PIs (Farthing *et al.*, 2006; Hill and Moyle, 2006). Such data show that both TPV and DRV are of useful option 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

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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.

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# **HIV-1 Integrase Inhibitors: Update and Perspectives**

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## **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.

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 ---

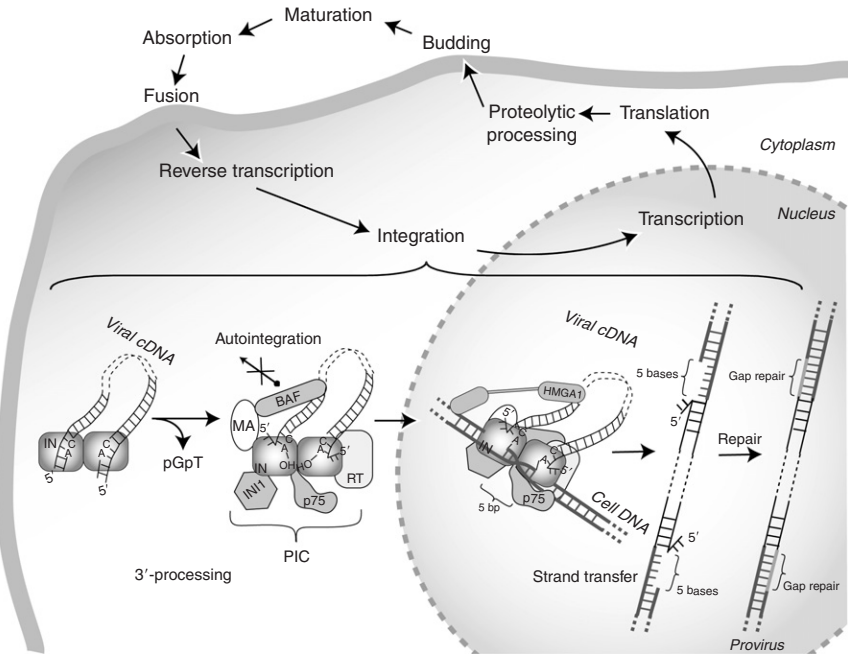
The encouraging results reported for two IN inhibitors in clinical trials and the recent insights in the cellular cofactors of DNA integration have renewed interest in HIV IN pharmacology and cellular biology. The current chapter gives an overview of the functions of IN and the cofactors for integration. 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

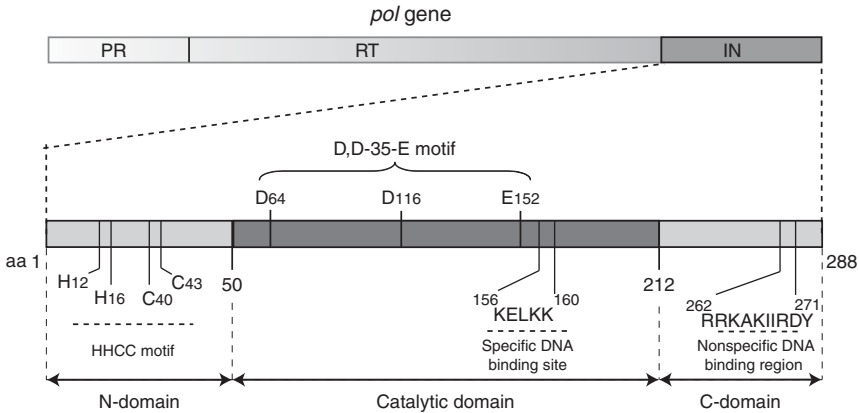


**FIGURE I** Integration in the HIV life cycle. IN: integrase, PIC: preintegration complex, BAF: barrier-to-autointegration factor, MA: HIV matrix protein, INI1: IN interactor 1, LEDGF/p75: lens epithelium-derived growth factor/transcription coactivator p75, RT: HIV reverse transcriptase, and HMGA1: 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



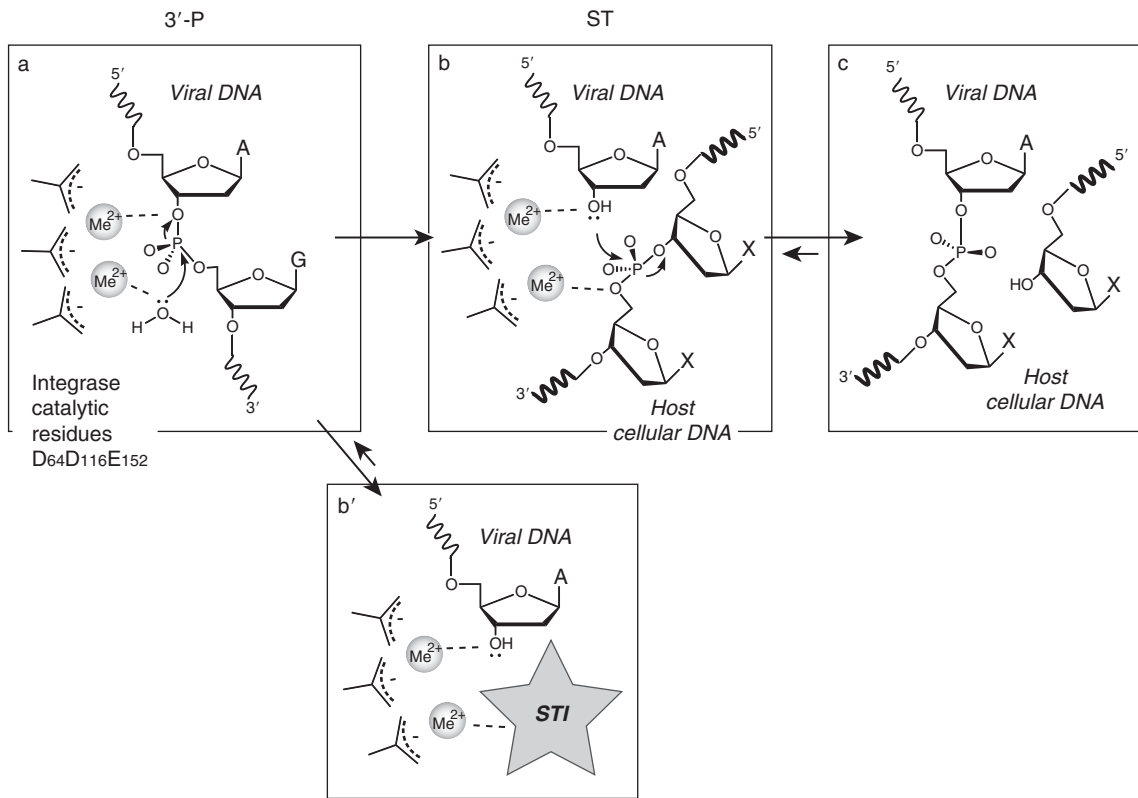
**FIGURE 2** Functional domains of HIV-1 integrase. IN: integrase, RT: HIV reverse transcriptase, PR: HIV protease.

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 I** Catalytic activities of HIV-1 IN mutants<sup>a</sup>

<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>	<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>
H12A;N*	+	+	+++	- (-)	PIC (1-4)	C56S/C65S/C130S/ C280S	++	-	+++		(13)
H12C*			+++	(-)	(5,6)	C56S/C65S/Q148C/ C280S	++	-			(13,15)
H12N/H16N	-	-	+		(2)	Q62E	++	++	+++		(16)
H12C/H16C			+++		(5)	Q62K				-	(11)
H12Q/H16Y				(-)	(7)	Q62N	+	-	-		(17)
Y15A				(-)	(8)	D64C;R*			-		(17)
H16A*				-	(3)	D64A;E;N;V*	-	-	-	-	(1,3,5,6,9, 18,19)
H16C;V*	+++	+++	+++	(-)	(5,6)	D64A/D116A				-	(3)
K34A	++	++		- (-)	PIC (4)	D64R/D116R			-		(17)
K46A				+++	(4)	D64A/E152A				-	(3)
C40A;S*	-	-	+	(-)	(1,9)	D64A/D116A/ E152A				-	(3)
C40S/C43S	-	-	++		(1)	C65S*	+++	+++		+++	(13,14)
D41A/K42A				+	(3)	C65A*				+++	(12)
C43A;S;L*	+	+		(-)	(7,9,10)	C65S/C130S				-	(13)
M50A				+	(9,10)	T66A*	+	++	++	(++)	(17,20)
H51A*				+	(3)	T66I*	++/+++	++/+++	++	+/+++	L-708,906; L-731,988; S-1360; 118D-24; L-CA (21-27)
H51A/D55V				-	(3)	T66I/L74M	+	+		+	L-708,906; S-1360; (23)
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)						

T66I/S153Y				++	L-708,906; L-731,988; 118D-24; L-CA (24-26)	T115A;S D116A;E;I;N*  D116C* D116A/E152A**	+++ -  - -	+++ -  +	+++ -  -	+++ - (-)	(1,9,18,31,32) (1,5,6,9,17, 18, 31-34) (17) (3)
T66I/M154I	++	++	++	+	L-731,988; L-CA (25)	D116/A23V** D116/E11D	- -	++ ++	- -	-	(35) (35)
T66I/L74M/S230R	+	+		++	S-1360; L-708, 906 (23)	D116/L28R/C65S/ T210N/L213I N117K;Q N117S	- +	+	-	-	(35)  (1,11,17,32,33) (17)
H67E*				Del	(11)	G118A	++ +	++ +	++ +	Del	(17)
H67S*	+++	+++	++		(17)	S119T;G;A;K	+++	+++	+++	+++	(32,33)
H67Q/K71E				-	(11)	N120Q;S*	+++	+++	+++		(29)
E69A/K71A				-	(3)	N120L;L;E;G*	+++	+++	+++		(17)
K71E*				+++	(11)	N120L;K*				(-)	(6)
V72I					L-870,810 (26)	N120L/Q148K				Del	(11)
L74M*	+++	+++			L-708,906; S-1360 (23,27)	F121A F121Y	+/- -	-		-	(11) (31) 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)	C130G*				- (-)	OH-Coum (12,36)
T93A				(++)	(20)	C130S*				-	OH-Coum (13,14,36)
G106A	+++	+++			(29)						
P109A;S*	+/-	+/-	-	+	(3,18,30)						
P109S/T125A	+++	+++	+++	(+/-)	(30)						
T112A	+++	+++			(18)	C130A/F185K/ C280S	+++	+++			OH-Coum (36)
H114E				++	(11)						

(Continues)



**Table I (Continued)**

<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>	<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>
C130S/F185K/ C280S	+++	+			OH-Coum (36)	N144K				-	(11)
W131G/F185K/ C280S	++	++			(36)	N144Q				-/(-)	(39)
W132A;G;R// F185K/C280S	+++	-			OH-Coum (36)	P145I*	-	-	-	+	(11)
W132Y/F185K/ C280S	+++	++			OH-Coum (36)	P145F*				-/(-)	(39)
I135P				(-)	(9)	P145I/F185K/C280S	-	-	-		(28)
K136A*	+/-	+/-	+++	-/++	(3,11,16)	Q146K				+	S-1360 (11,27)
K136E;R*	+++	+++	+++	+++	(16)	S147I	-		+++	-	(32,33)
K136A/E138A	+++	+++	+++	+++	(16)	Q148A*	++	-			(15)
K136R//F185K/ C280S	+++	++			(36)	Q148K*				Del	(11)
E138A*				+	(3)	Q148L*	+	++	+	Del	(11,17)
E138K*				++	S-1360 (11,27)	Q148N*	+++	++			(15)
G140S*	+	+	+	Del	L-CA; L-731,988; (37)	Q148A/F185K/ C280S	+	+			(36)
G140S/F185K/ C280S	+++	+++	+++		(38)	V150E	+++	+++	+++		(28)
P142F				+++/ (+++)	(39)	V151A*				(+)	(9)
Y143F				Del	(3,32)	V151I*					L-870,810 (26)
Y143N	+++		+++	Del	(3,32,33)	V151D/E152Q	-	-	-	-	(10)
Y143G				+++/ (+++)	(11,20,39)	V151T/S153Q	++	-	+++		(40)
						V151L/S153L	+++	-	-		(40)
						V151L/E152V/ S153F	++	-	-		(40)
						V151A/E152M/ S153A	++	-	-		(40)
						V151Y/E152V/ S153P	+++	-	-		(40)

V151H/E152S/ S153T	+++	-	-		(40)	N155L N155S	+	+	-	Del	(11,17)
V151A/E152L/ S153T	+++	-	-		(40)	K156A*					L-870, 810 (26)
V151K/E152L/ S153T	++	-	-		(40)	K156E*	-	-	+/-	+ / + + + / (+++)	(3,39)
V151S/E152S/S153P	++	-	-		(40)	K156R*				Del	(11,17,41)
V151H/E152G/ S153L	+++	-	-		(40)	K156I*	-	-	-		(42)
V151E/E152S/ S153N	++	-	-		(40)	K156E/K159E	-	-	-	-	(28)
V151T/E152S/ S153M	+++	-	-		(40)	K156A/E157A E157A/K159A				+	(11,41)
V151T/E152F/ S153C	+++	-	-		(40)	L158F	+++	+++	+++	+	(3)
E152A;C;D;G;H;P; Q;V;K*	-	-	-	- (-)	(1,3,5,6,9, 11,17,18, 28,31, 33,34)	K159A*				++/(+++)	(28)
E152A/K156A				-	(3)	K159E*	-	-	+++	-/Del	(39)
E152N/S153R	++	-	-		(40)	K159N;S*	+	+	+		(17)
S153A;R*	+	+	+++	++	(1,11)	K159Q*	+++		+++		(33)
S153A*	+++		+++	+++	S-1360	K159R*					(42)
S153Y*	++	++	++	+	L-708,906; L-731,988; L-CA (22,25,26)	K159A/K160A				+	(3)
M154I	+++	+++	+++	++	L-708,906; L-731,988; L-CA (22,25,26)	K159A;P;Q				(+)	(9,31,32)
N155E;K	-	-	-	Del	(11,17)	K160A*				+++ / (+++)	(39)
						K160D*					S-1360 (27)
						K160E*	+++	+++	+++	-	(41)
						V165A	++			- (-)	PIC (4,12)
						V165I					S-1360 (27)
						R166A*				-	(3)
						R166T*	++	++			(42,43)
						R166A/D167A				-	(3)
						D167A*				+	(3)
						Q168A	+++	+++		(-)	LEDGF (44,45)
						Q168L	+++	-		(-)	LEDGF (44)
						Q168P	-	-		(-)	LEDGF (44)

(Continues)

**Table I (Continued)**

<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>	<i>Mutations</i>	<i>3'-P</i>	<i>ST</i>	<i>Dis</i>	<i>Inf</i>	<i>Interaction (References)<sup>b</sup></i>
E170A/H171A	+++	+++		+/Del	(3,46)	N222A	++	++	++		(47)
E170A/K173A				+	(3)	F223A	++	++	++		mAb33
H171A/K173A*				+	(3)						(47,48)
L172M*	+++	+++	+++		(28)	R224A	++	++	++		mAb33
L172A/K173A	+++	+++		-	(46)						(47,48)
T174A	+	+		-	(46)	Y226					mAb33
V176A/Q177A	+++	+++		Del	(46)						(48)
M178A	-	-		-	(46)	Y227A	++	++	++		(47)
A179P				(-)	(9)	R228A					(49)
V180A/F181A	-	-		-	(46)	S230R*	+++	+++			L-708,906; S-1360 (23)
N184D;L				-	(11)						
F185A;K;L;H*	+++	+++		(-)	(16)	R231A	++	+	+	+	(47,49)
K186A;Q;E	+++			- (-)	(12,20)	P233A	++	++	++		(47)
K187A				-	(12)	L;V234A	++	++	++	+++	(47,49)
K188A				-	(12)	W235A;E;F	+++	+++	+++	+/- (-)	PIC (2, 4-6,9)
G189A	+++	+++			(18)						
S195A				(++)	(20)	K236A*				+++	(49)
E198A/R199A				-	(3)	K236E*				- (-)	(49)
R199A;C*	+++	+++	+++	-/Del	(3,5,6, 11,12)	K236A/K240A				-	(3)
				(-)		K236/E246A				- (-)	(49)
R199E*				-	(11)	L241A	-	-	-	- (-)	(47,49)
R199A/D202A				-	(3)	L242A	-	-	+	- (-)	(47,49)
R199T/D202A				-	(3)	W243A	++	++	++		(47)
V201I					(27)	K244A*				- (-)	(49)
K211A*				(++)	(20)	K244E*	-	-		(-)	(50)
K211A/E212A				+	(3)	K244A/E246A				-	(3)
Q214L/Q216L	+++			- (-)	(12)	E246A*	++	++	++	Del (+)	(47,49)
K215A*				+++	(12)	E246K*				- (-)	(49)
K215A/K219A	++			- (-)	(12)	D253A/D256A				+	(30)
K219A*				+	(12)	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)						

<sup>a</sup>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.

<sup>b</sup>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

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### 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

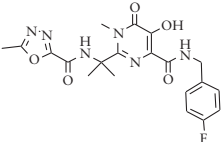
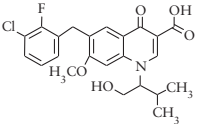
used to evaluate the site of drug action; as it is the only reaction that can be catalyzed by the IN catalytic core. Compounds that compete with target DNA within the enzyme catalytic site (Fig. 3b') produce preferential inhibition of ST over 3'-P and are generally ineffective against disintegration (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).

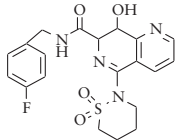
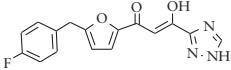
As it remains difficult to obtain drug-IN co-crystals, IN-DNA binding assays continue to be developed to investigate drug-binding sites in the IN-DNA complex. The Schiff base (Mazumder and Pommier, 1995) and disulfide cross-linking (Johnson *et al.*, 2006) assays can be used to determine whether a given 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 Cherfilis, 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 Cherfilis, 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				Comments	Clinical development status
		Anti-IN activity IC <sub>50</sub> , (μM)		Antiviral activity			
Name, source (references) <sup>a</sup>	Structure	3'-P	ST	EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)		
MK-0518, Raltegravir Insentress <sup>TM</sup> Merck & Co. (1,2,3,4)		<sup>b</sup>	0.016	0.017– 0.029 (EC <sub>95</sub> )	<sup>b</sup>	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 <sup>-4</sup>	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 <sub>95</sub> )	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 <sup>c</sup> (11,12,13)		0.02 <sup>d</sup>		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

<sup>a</sup> 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).

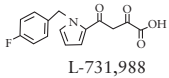
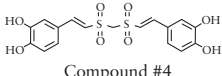
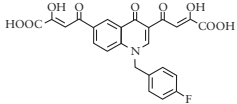
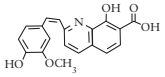
<sup>b</sup> Data unavailable from references.

<sup>c</sup> Joint venture Shionogi-GlaxoSmithKline Pharmaceuticals.

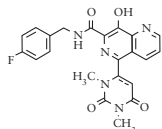
<sup>d</sup> No information regarding selectivity for 3'-P or ST.



**TABLE III** Representative Inhibitors of HIV-1 IN

Chemical family	Structure	Anti-IN activity $IC_{50}$ ( $\mu\text{M}$ ) <sup>a</sup>		Antiviral activity		Comments (references) <sup>b</sup>
		3'-P	ST	$EC_{50}$ ( $\mu\text{M}$ )	$CC_{50}$ ( $\mu\text{M}$ )	
Diketo acids (DKA)	 L-731,988	6	0.08	1	<sup>c</sup>	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)	ILPWKWPWWPWR Indolicidin	60	57	(35–52 $\mu\text{M}$ )	<sup>c</sup>	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

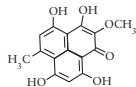
<sup>c</sup>

0.035

0.02–0.04 (EC<sub>95</sub>) <sup>c</sup>

Good pharmacokinetics and oral bioavailability when dosed in rats and dogs (9)

Natural products



Funalenone

<sup>c</sup>

10

1.7

87

Isolated from *Penicillium* sp. FKI-1463 (10)

<sup>a</sup> Abbreviations: IC<sub>50</sub>, concentration required for 50% inhibition of HIV-1 integrase activity; EC<sub>50</sub>, concentration required to induce the exponential growth of MT-2 cells infected by HIV by 50%; CC<sub>50</sub>, cytotoxicity of compound.

<sup>b</sup> 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).

<sup>c</sup> 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

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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

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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),  $\alpha$ -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  $\alpha$ -hydroxytropolones (Budihis *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

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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 metabolic activation pathways (Laufer *et al.*, 2006). Finally, besides systemic therapies, topical IN inhibitors are worthwhile pursuing as curative and preventive therapies.

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

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## **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



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

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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 mucus, 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.

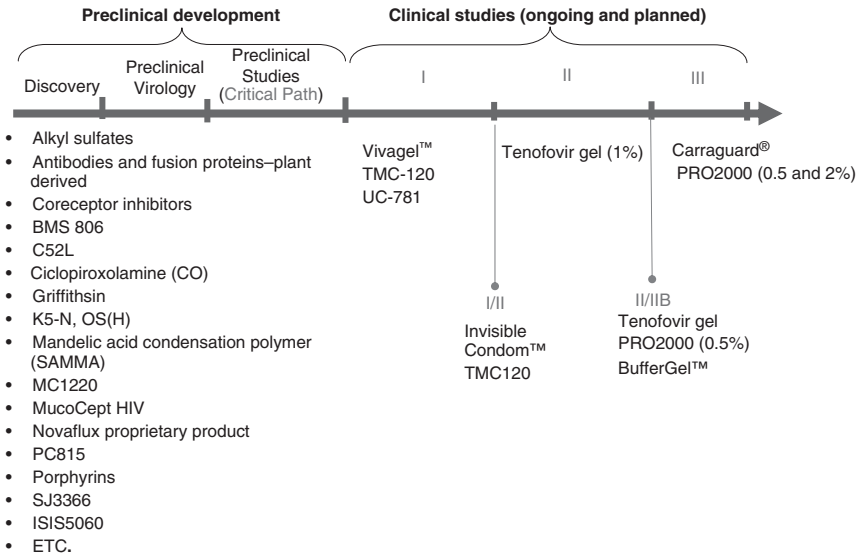
### III. Topical Microbicides in Preclinical Development

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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

<i>Property</i>	<i>Acceptable</i>	<i>Unacceptable</i>
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 I** Microbicide candidates in development. Adapted from summaries provided by the Alliance for Microbicide Development ([www.microbicide.org](http://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](http://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, Carraguard<sup>TM</sup>, and Buffer-Gel<sup>TM</sup>) 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

<i>Target</i>	<i>Subtarget</i>	<i>Candidates</i>
Virus	Nonspecific virucidals Specific virucidals	Detergents, metal ions NCp7 inhibitors, Autocatalytic antibodies
Virus entry	Virus–cell interaction gp120-CD4 Gp41 Coreceptor	Selectins, integrins oligosaccharides Antibodies, BMS806 C52L, T1249 CMPD167, TAK779
Reverse transcriptase	NNRTI NRTI*	TMC120, UC781 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<sup>TM</sup> (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.

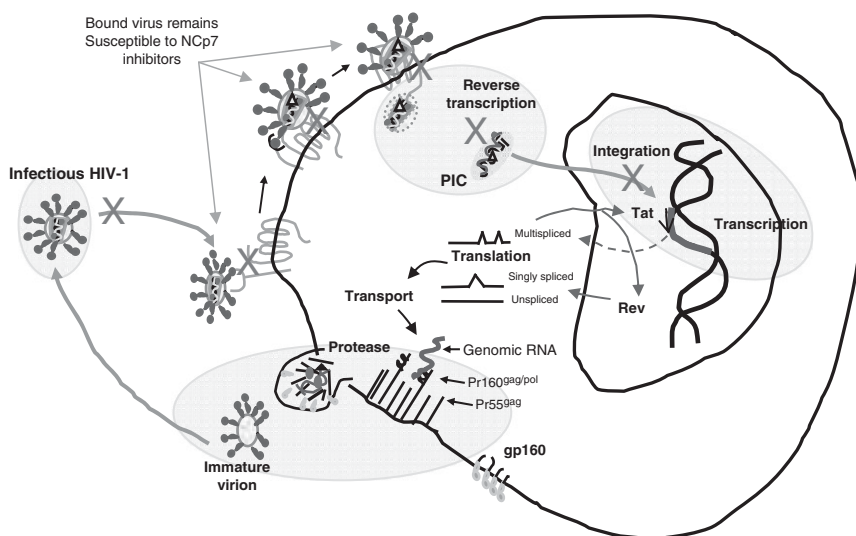
The Bill and Melinda Gates Foundation sponsored the Microbicide Development Strategy (MDS) in order to assure that microbicide development

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





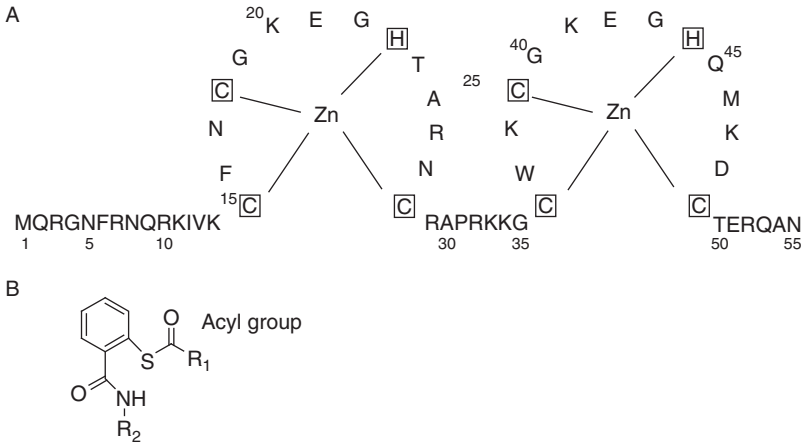
**FIGURE 2** HIV-1 replication cycle. Xs indicate potential targets for topical microbicide intervention. Grayed areas identify segments in the replication cycle indicating stages where the HIV NCp7 or its zinc fingers play critical roles in HIV replication. These areas represent potential targets for 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<sub>2</sub>-Cys-Xaa<sub>4</sub>-His-Xaa<sub>4</sub>-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

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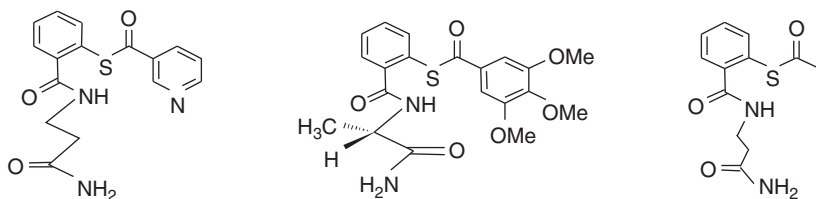
Members of the SAMT chemotype (Fig. 3B) were investigated for a number of critical properties in terms of their candidacy as potential NCp7-targeted antivirals, including antiviral efficacy, cellular toxicity, and chemical properties. In general, the SAMT compounds inhibited HIV replication in both acutely and chronically HIV-infected *in vitro* cell models. These compounds demonstrated reduced *in vitro* cytotoxicity, improved aqueous solubility, nonsusceptibility 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  $\mu\text{M}$ ). 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  $\text{Zn}^{2+}$  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/creatinine 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  $\mu M$ ) of Lead SAMTs

	19	89	247	AZT	AT2	DS (mg/ml)
CEM-SS/RF <sup>a</sup>	2.9	2.1	2.5	0.004	—	—
PBMC/B subtype <sup>b</sup>	1.1	0.02	ND	0.007	—	—
Mono/BaL <sup>b</sup>	5.0	<0.01	ND	0.01	—	—
PBMC/HIV-2 <sub>CDC310342</sub> <sup>b</sup>	0.5	5.9	ND	0.002	—	—
PBMC/SIV <sub>Mac251</sub> <sup>b</sup>	1.7	4.7	ND	0.018	—	—
PBMC/MDR-769 <sup>b</sup>	1.2	4.2	ND	0.6	—	—
Cobalt ejection <sup>c</sup>	35.9	15.4	29.6	—	—	—
Virucidal <sup>d</sup>	9.0	12.0	ND	—	2.2	—
Transmission:						
Syncytia <sup>e</sup>	0.6	0.9	ND	—	—	0.8
Replication <sup>e</sup>	0.08	0.1	ND	—	—	0.4

<sup>a</sup>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).

<sup>b</sup>Viral replication were determined on day 7 PI by measuring supernatant RT activity or p24 antigen expression by ELISA.

<sup>c</sup>Cobalt was substituted for zinc to compare metal ion ejection rates of the compounds. Values represent milli-absorbance units.

<sup>d</sup>Reduction in infectivity of HIV-1 IIIB following a 4-h incubation with thioesters. Residual infectivity was determined in HeLa CD4 LTR  $\beta$ -gal cells by chemiluminescence.

<sup>e</sup>CEM-SS cells chronically infected with the SK-1 strain of HIV-1 were assessed for supernatant RT or syncytia formation.

Units are in  $\mu M$  unless stated otherwise.

AT2, alderthiol-2; DS, dextran sulfate ( $\mu g/ml$ ); ND, not done; PBMC, peripheral blood mononuclear cell.

function linked to amino acid primary amides, namely, those of glycine,  $\beta$ -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 ---

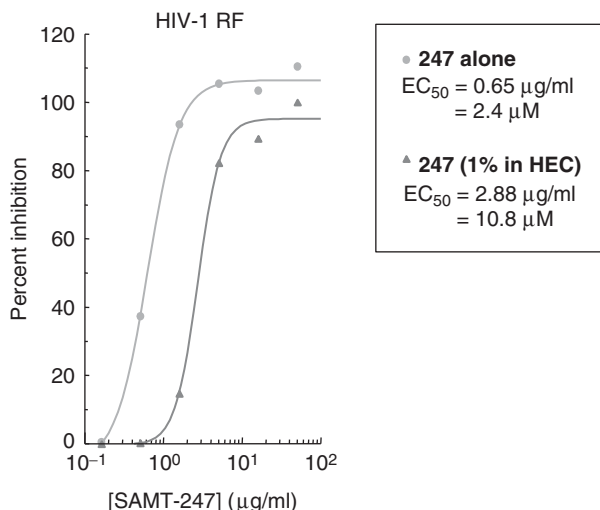
In order to determine if the thioester compounds were potential microbicide candidates, we initially tested the SAMTs for their ability to prevent cell-to-cell transmission of HIV-1. [Table III](#) shows that two of the lead SAMT analogs were able to potently prevent the transmission of HIV from chronically 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-1 $\beta$  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/IB 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  $\mu$ M vs 1% suspension 10.8  $\mu$ M). 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.

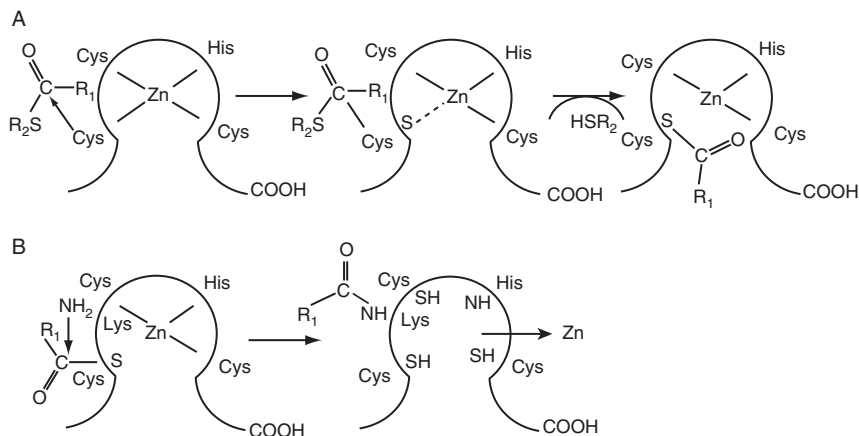
The next logical extension of our efforts to determine whether SAMT are potential topical microbicide candidates was to determine if the SAMT could prevent vaginal transmission of SHIV in monkeys. Using the X4 and R5 dual infection model [CXCR4 (X4) SHIV<sub>SF33A</sub> and CCR5 (R5) SHIV<sub>SF162P3</sub>] 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<sub>50</sub> (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  $\text{Co}^{2+}$  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

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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

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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 identified 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.

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# Viral Drug Resistance and Fitness

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## I. Chapter Overview

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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

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

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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 inpatient 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

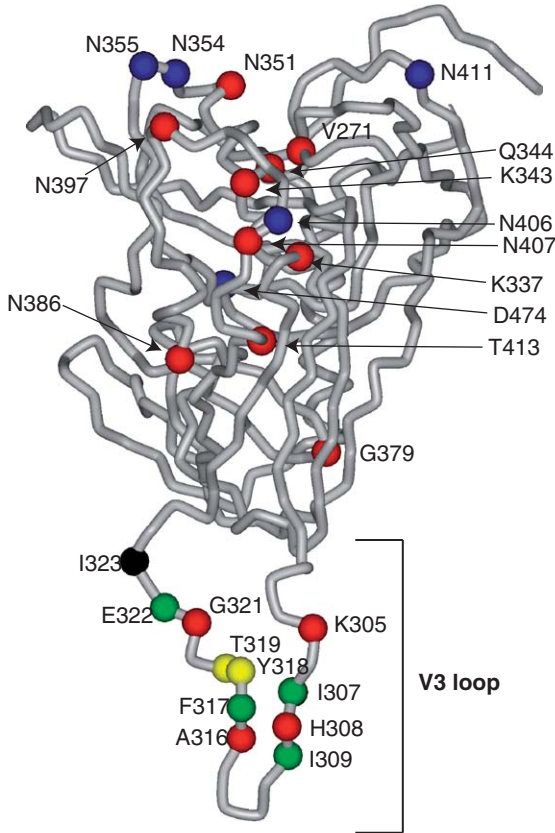
the entire virus or if the M184V virus rapidly compensates for this defect. In addition, this review has focused on emerging studies with EIs in relation to drug resistance and fitness. Several recent reviews have carefully addressed the fitness impact of resistance to PIs and RTIs.

## A. Entry Inhibitors

### I. 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-1 $\beta$  and RANTES, but not MIP-1 $\alpha$ , 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<sub>JR-FL</sub> 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<sub>JR-FL</sub> 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<sub>BAL</sub>. However, when primary HIV-1 viruses were assessed, three out of six viruses developed resistance (two patient isolates and HIV-1<sub>SF162</sub>). 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<sub>SF-162</sub> 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 (Quiñones-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-1 $\alpha$  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<sub>50</sub> 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.

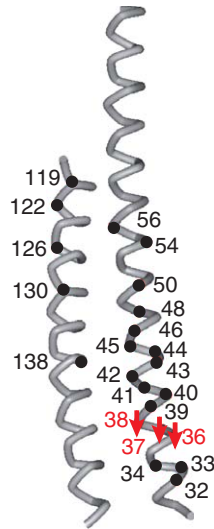
**b. Attachment Inhibitors** Drug resistance has been described for three different attachment inhibitors. Cyanovirin N (CV-N) targets the high mannose glycans in gp120 as does concanavalin A (ConA), but the ConA has a lower specificity for high mannose glycans than CV-N (Witvrouw *et al.*, 2005). Escalating the dose of these lectins in the presence of replicating HIV-1<sub>NL4-3</sub>, 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 passing HIV-1<sub>NL4-3</sub> and HIV-1<sub>LAI</sub> 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<sub>50</sub>) 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 (Desmeziers *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 (Desmeziers *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 ( $\Delta$ FNSTW) 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<sub>NL4-3</sub> 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<sub>NL4-3</sub> 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<sup>+</sup> 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, apliviroc, 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 derivatives (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**

### ***I. 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).

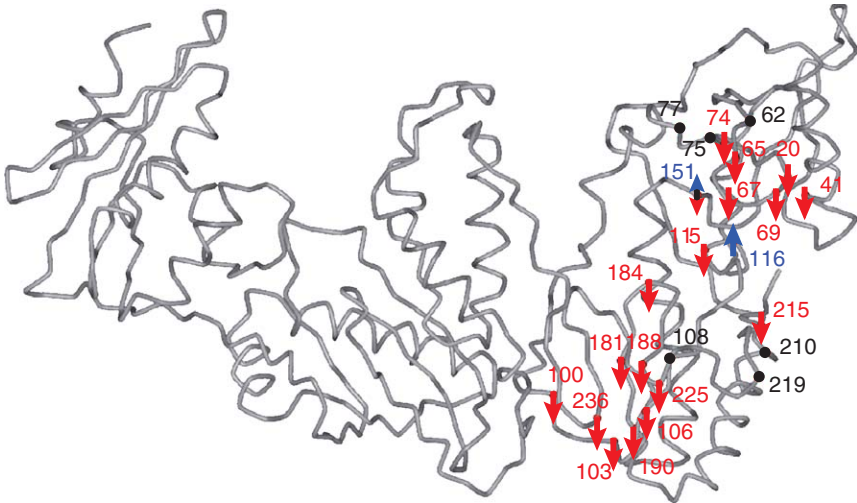


NRTIs are administered as prodrugs, which require host cell entry and triphosphorylation (Furman *et al.*, 1986; Hart *et al.*, 1992; Mitsuya and Broder, 1986; Mitsuya *et al.*, 1985; St Clair *et al.*, 1987) by cellular kinases prior to enacting an antiviral effect. Lack of a 3'-hydroxyl group at the sugar (2'-deoxyribose) 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-triphosphate 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 disoproxil 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/E, and K219Q/E (Bachelier *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 (Bachelier *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 (Rogers *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

### I. 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 (Bachelier *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; Quiñones-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.

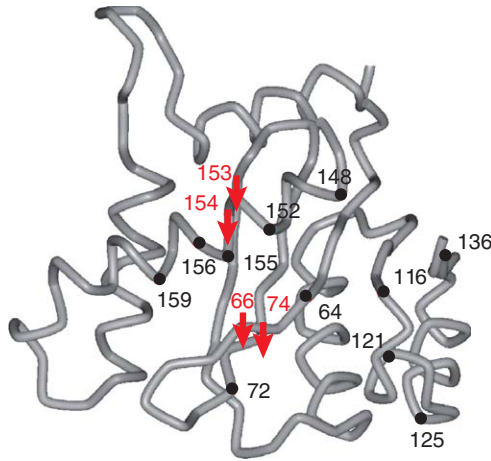
## D. Integrase Inhibitors

### I. 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



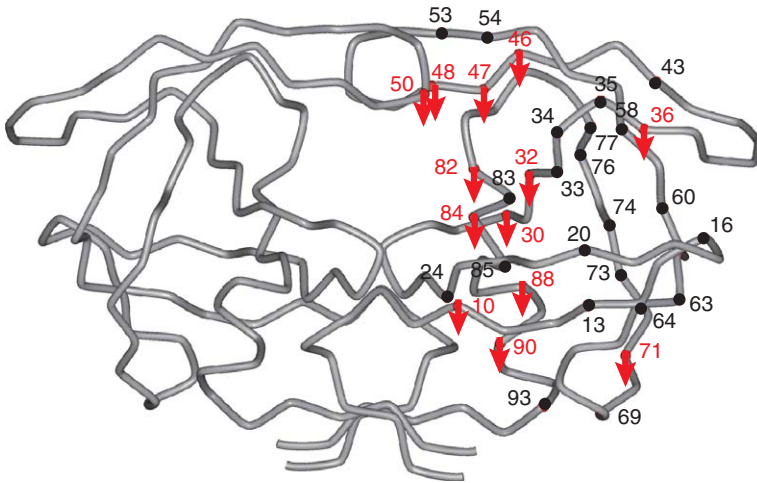
was associated with a decrease in the replication kinetics when compared with the wild-type HIV-1 strain. Similar results were obtained during *in vitro* passage of HIV-1<sub>IIIB</sub> in the presence of increasing concentrations of the diketo analogue S-1360 (Fikkert *et al.*, 2004). A total of nine 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

### I. 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 brexanavir (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 brexanavir, 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<sup>gag</sup> 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-1<sub>NL4-3</sub> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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.

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

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## I. Chapter Overview

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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

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 TRIM5 $\alpha$ ) 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 <sup>+</sup> 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<sup>+</sup> Cells Resistant to HIV-1 Infection

<i>Therapeutic gene</i>	<i>Vector</i>	<i>Target cells</i>	<i>References</i>
<i>RevM10</i> (dominant negative <i>Rev</i> mutant)	Plasmid transfection using gold microparticles	CD4 <sup>+</sup> T lymphocytes	Woffendin <i>et al.</i> , 1996
	Retrovirus	CD4 <sup>+</sup> T lymphocytes	Ranga <i>et al.</i> , 1998
		CD34 <sup>+</sup> bone marrow cells	Podsakoff <i>et al.</i> , 2005
		Mobilized allogenic CD34 <sup>+</sup> cells from peripheral blood	Kang <i>et al.</i> , 2002
<i>RevM10</i> and/or <i>RevM10</i> plus antisense <i>TAR</i>	Retrovirus	CD4 <sup>+</sup> T lymphocytes from HIV-negative identical twins	Morgan and Walker, 1996; Morgan <i>et al.</i> , 2005
RRE decoy	Retrovirus	CD34 <sup>+</sup> bone marrow cells	Kohn <i>et al.</i> , 1999
Antisense RNAs against <i>TAR</i> and <i>Tat/Rev</i> (HGTV43)	Retrovirus	CD34 <sup>+</sup> bone marrow cells	
Long antisense against <i>env</i> , driven by the HIV-1 LTR (VRX496)	Lentivirus	CD4 <sup>+</sup> T lymphocytes	Levine <i>et al.</i> , 2006; MacGregor, 2001
Hairpin ribozyme against HIV-1 <i>U5</i> leader sequence	Retrovirus	CD4 <sup>+</sup> T lymphocytes	Looney <i>et al.</i> , 1998
Hammerhead ribozyme targeted to <i>Tat</i> and <i>Rev</i>	Retrovirus	CD34 <sup>+</sup> bone marrow cells	Krishnan <i>et al.</i> , 2002; Michienzi <i>et al.</i> , 2003; Zaia, 2003
		CD34 <sup>+</sup> bone marrow cells after myeloablation	Krishnan <i>et al.</i> , 2002; Michienzi <i>et al.</i> , 2003; Zaia, 2003
Hammerhead ribozyme against the translation initiation region of <i>tat</i> (Rz2, OZ1)	Retrovirus	CD4 <sup>+</sup> T lymphocytes from HIV-negative identical twins	Macpherson <i>et al.</i> , 2005
		Mobilized CD34 <sup>+</sup> cells from peripheral blood	Amado <i>et al.</i> , 1999, 2004
Gp41-derived peptide blocking fusion (M87o)	Retrovirus	CD4 <sup>+</sup> T lymphocytes	Egelhofer <i>et al.</i> , 2004
<i>TAR</i> decoy RNA, siRNA against <i>Tat</i> and <i>Rev</i> , ribozyme against cellular <i>CCR5</i>	Lentivirus	CD34 <sup>+</sup> bone marrow cells	Li <i>et al.</i> , 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 ( $\approx 9$  kb) and singly spliced ( $\approx 4$  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<sup>+</sup> 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<sup>+</sup> cells has also been confirmed by an additional experimentation involving retrovirus-mediated gene transfer into CD34<sup>+</sup> 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 ( $\leq 1$  every  $1 \times 10^6$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> hematopoietic progenitor cells (Bauer *et al.*, 1997). Following these observations, four HIV-1-infected children and adolescents underwent bone marrow harvest from which CD34<sup>+</sup> 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  $1 \times 10^5$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T lymphocytes in identical twins discordant for infection or autologous mobilized peripheral blood CD34<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>4</sup> to 1/10<sup>5</sup> of hematopoietic cells analyzed, including naïve T cells. Since the CD4<sup>+</sup> 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**

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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<sup>+</sup> T lymphocytes; (2) that this task will be only feasible when improved conditions for gene transfer of CD34<sup>+</sup> 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  $\gamma$ -retrovirus-mediated gene transfer of normal cDNAs into autologous CD34<sup>+</sup> HSCs (Kohn *et al.*, 2003). The overall results of these trials have indicated that *ex vivo* gene transfer with  $\gamma$ -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  $\gamma$ -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  $\gamma$ -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<sup>+</sup> 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<sup>+</sup> 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  $\gamma$ -retroviral vector was used to infect purified CD34<sup>+</sup> 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-EVII* 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  $\gamma$ -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  $\gamma$ -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<sup>+</sup> 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](http://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  $\gamma$ -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  $\gamma$ -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  $\gamma$ -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 erythroleukemia, 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<sup>+</sup> 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 acids 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 Droscha (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; Sabariego *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<sub>HXB2</sub> 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<sup>+</sup> 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-1 $\alpha$  (MIP-1 $\alpha$ ), 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 intrakinins (modified RANTES and MIP-1 $\alpha$  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 Banerjee, 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?**

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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<sup>+</sup> 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<sup>+</sup> 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 myeloblastic regimens (which favors engraftment of the *ex vivo* modified cells) will be likely to significantly increase the number of resistant CD4<sup>+</sup> T cells that will ultimately be generated *in vivo*. The recent CDG trial indicates that  $\gamma$ -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 TRIM5 $\alpha$ , 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 trials 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.

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

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## I. Chapter Overview

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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

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

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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, metabolome.

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

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#### 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

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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 TFIIF (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<sup>a</sup>

<i>Viral protein category</i>	<i>HIV-1 virus protein</i>	<i>Cellular-interacting proteins</i>
Structural	Gag (p55)	
	p17—Matrix	BAF (Lin and Engelman, 2003; Mansharamani <i>et al.</i> , 2003), Calmodulin (Daube <i>et al.</i> , 1991; Radding <i>et al.</i> , 2000), HEED (Peytavi <i>et al.</i> , 1999), EF1 $\alpha$ (Cimarelli and Luban, 1999), hIF2 (Wilson <i>et al.</i> , 1999), Actin (Bukrinskaya <i>et al.</i> , 1998)
	p24—Capsid	CypPA (Luban <i>et al.</i> , 1993)
	p7—Nucleocapsid	Actin (Cimarelli and Luban, 1999; Liu <i>et al.</i> , 1999)
	p6	Tsg101 (Garrus <i>et al.</i> , 2001; VerPlank <i>et al.</i> , 2001), AIP1 (Strack <i>et al.</i> , 2003)
	Pol	
	p51—Reverse transcriptase	$\beta$ -Actin (Liu <i>et al.</i> , 1999)
	p31—Integrase	Importin/Karyopherin $\alpha/\beta$ (Gallay <i>et al.</i> , 1997; Hottiger and Nabel, 1998), INI1 (Gallay <i>et al.</i> , 1997), UDG (Yung <i>et al.</i> , 2001)
	Env (gp160)	ApoH (Willets <i>et al.</i> , 1999)
	gp120	CD4 (McDougal <i>et al.</i> , 1986)
Accessory	Vpu (p16)	BTrCP (Margottin <i>et al.</i> , 1998), UBP (Callahan <i>et al.</i> , 1998), Fas (Casella <i>et al.</i> , 1999)
	Vpr (p12/p10)	TFIIB (Agostini <i>et al.</i> , 1996), Cyclin T1/CDK9 (Sawaya <i>et al.</i> , 2000), hVIP/mov34 (Mahalingam <i>et al.</i> , 1998), Karyopherin $\alpha$ (Gallay <i>et al.</i> , 1997), ANT (Jacotot <i>et al.</i> , 2001), HHR23A (Withers-Ward <i>et al.</i> , 1997), p300 (Felzien <i>et al.</i> , 1998), p53 (Sawaya <i>et al.</i> , 1998), UNG (Bouhamdan <i>et al.</i> , 1996), RIP/VprBP (Zhang <i>et al.</i> , 2001)
	Vif (p23)	Sp140 (Madani <i>et al.</i> , 2002), Vimentin (Karczewski and Strebel, 1996), Cul5 (Yu <i>et al.</i> , 2003), Elongin B/C (Yu <i>et al.</i> , 2003), Rbx1 (Yu <i>et al.</i> , 2003), CEM15/APOBEC3G (Sheehy <i>et al.</i> , 2003)
	Nef (p27/p25)	NBP1 (Lu <i>et al.</i> , 1998), Human thioesterase II (Watanabe <i>et al.</i> , 1997), CD4 (Grzesiek <i>et al.</i> , 1996), ASK1 (Geleziunas <i>et al.</i> , 2001; Peterlin and Trono, 2003), AP-1, 2, 3 (Coleman <i>et al.</i> , 2006; Craig <i>et al.</i> , 2000; Greenberg <i>et al.</i> , 1997; Schwartz <i>et al.</i> , 1996), MHC-I (Schwartz <i>et al.</i> , 1996; Williams <i>et al.</i> , 2002), PI3K (Wolf <i>et al.</i> , 2001), $\beta$ -COP (Piguet <i>et al.</i> , 1999), PACS1 (Piguet <i>et al.</i> , 2000), Src family tyrosine kinases (Lee <i>et al.</i> , 1996), p53 (Greenway <i>et al.</i> , 2002)

(continued)

**TABLE I** (*continued*)

<i>Viral protein category</i>	<i>HIV-1 virus protein</i>	<i>Cellular-interacting proteins</i>
Regulatory	Tat (p16/p14)	TBP (Chiang and Roeder, 1995; Dal Monte <i>et al.</i> , 1997; Garcia-Martinez <i>et al.</i> , 1997; Kashanchi <i>et al.</i> , 1994; Roebuck <i>et al.</i> , 1997; Veschambre <i>et al.</i> , 1995), p32 (Fridell <i>et al.</i> , 1995), CBP/p300 (Col <i>et al.</i> , 2001; Deng <i>et al.</i> , 2000, 2001; Hottiger and Nabel, 1998; Kiernan <i>et al.</i> , 1999; Ott <i>et al.</i> , 1999), CAK/TFIIH (Blau <i>et al.</i> , 1996; Cujec <i>et al.</i> , 1997; Herrmann and Mancini, 2001; Parada and Roeder, 1996), Tip60 (Kamine <i>et al.</i> , 1996), P-TEFb (Chen <i>et al.</i> , 1999; de Falco and Giordano, 1998; Fujinaga <i>et al.</i> , 1998; Herrmann and Mancini, 2001; Karn, 1999; Majello <i>et al.</i> , 1999; Wei <i>et al.</i> , 1998), Sp1 (Chun <i>et al.</i> , 1998; Kamine <i>et al.</i> , 1991), BRG1 (Agbottah <i>et al.</i> , 2006; Mahmoudi <i>et al.</i> , 2006; Mohrmann <i>et al.</i> , 2004)
	Rev (p19)	p32 (Tange <i>et al.</i> , 1996), eIF-5A (Ruhl <i>et al.</i> , 1993), CRM1 (Neville <i>et al.</i> , 1997), hRIP/Rab (Farjot <i>et al.</i> , 1999), Importin- $\beta$ (Truant and Cullen, 1999), B23 (Fankhauser <i>et al.</i> , 1991)

<sup>a</sup>Abbreviations: BAF, barrier-to-autointegration factor; HEED, human EED; EF1 $\alpha$ , elongation factor-1 $\alpha$ ; 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-EFb, phosphorylated positive transcription elongation factor b.

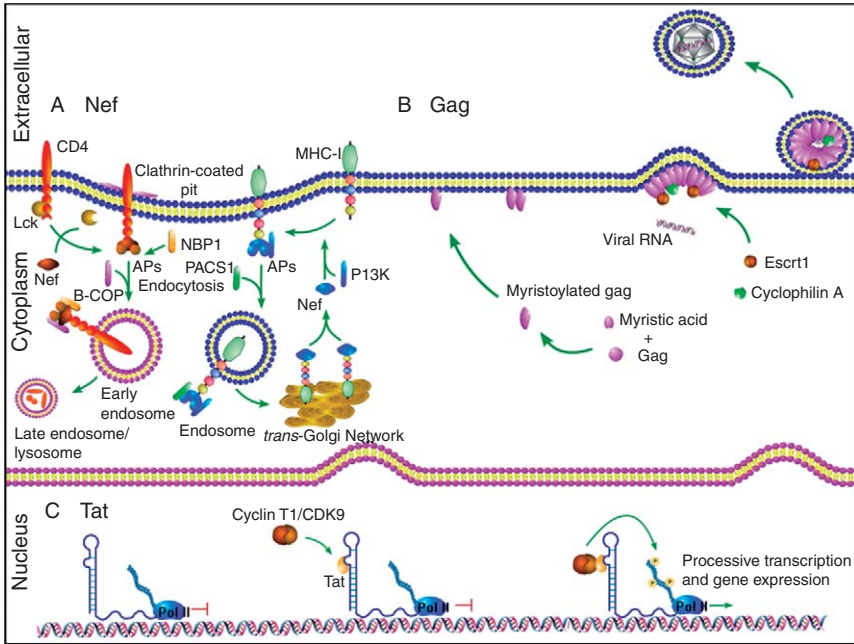


elongation factor b (P-TEFb)], emerging work indicates the importance of Tat in recruiting other factors required for initiation and elongation. Several reports have indicated a role of Tat in associating with TBP and facilitating transcriptional initiation (Brady and Kashanchi, 2005; Chiang and Roeder, 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).

### **I. 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 II CTD 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 I** 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  $\beta$ -COP1, which binds to CD4-bound Nef, facilitated by binding of Nef-binding protein-1 (NBP1).  $\beta$ -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 Esrc1, 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.

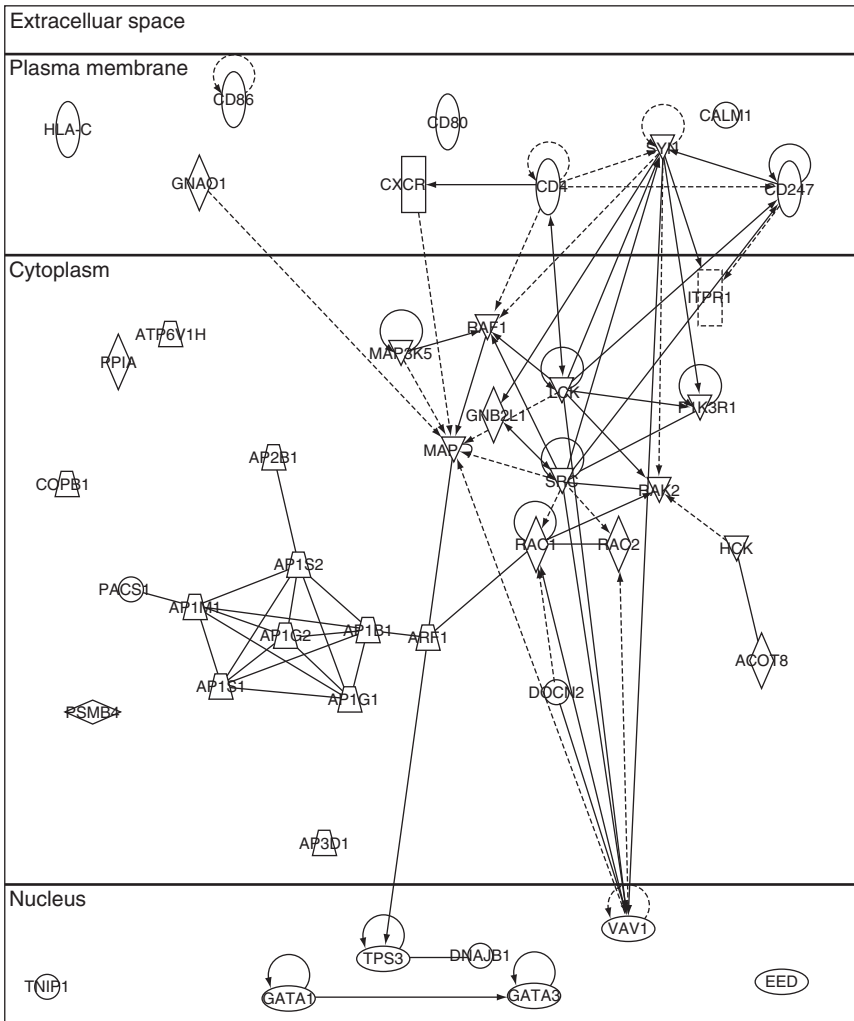
## B. Cellular Protein Interactions of Nef

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).

### **I. 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  $\beta$ -COP, the  $\beta$ -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).

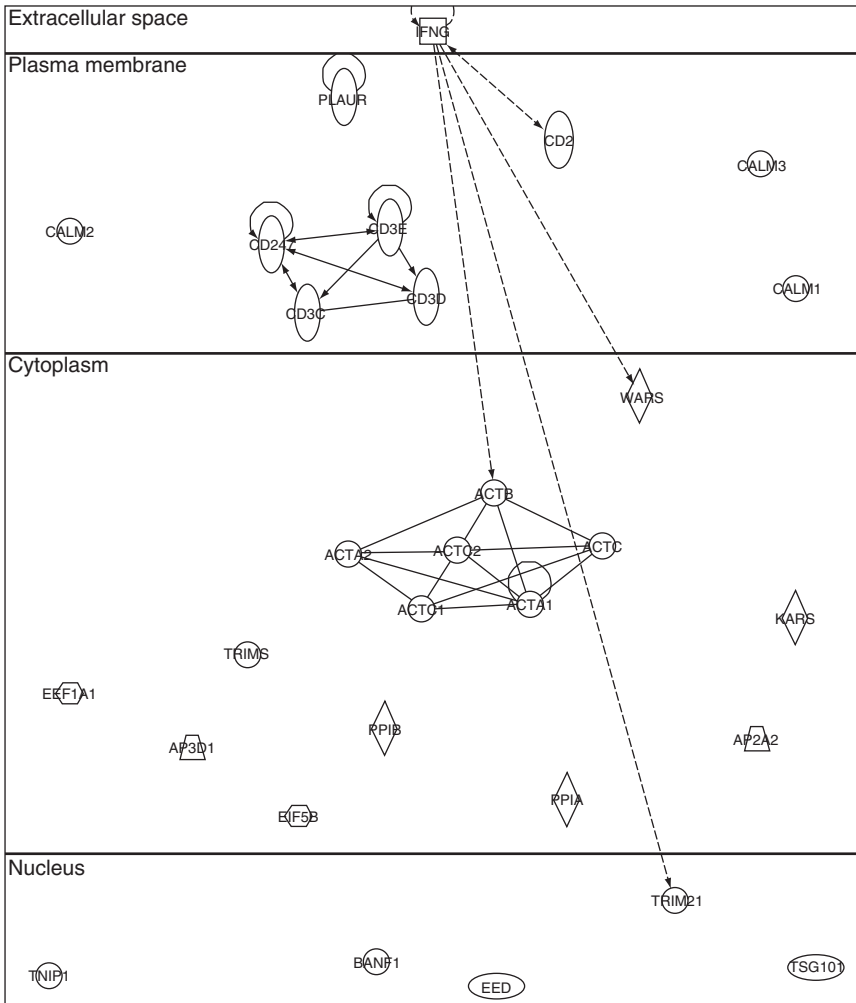
### 3. Nef and Apoptosis

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- $\alpha$ -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<sub>L</sub> 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<sup>Gag</sup>, 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<sup>Gag</sup> 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-1 $\alpha$ , 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

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### 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- $\gamma$ , 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<sup>+</sup> 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  $\beta$ -actin and  $\beta$ -tubulin. In the absence of  $\beta$ -actin and  $\beta$ -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.

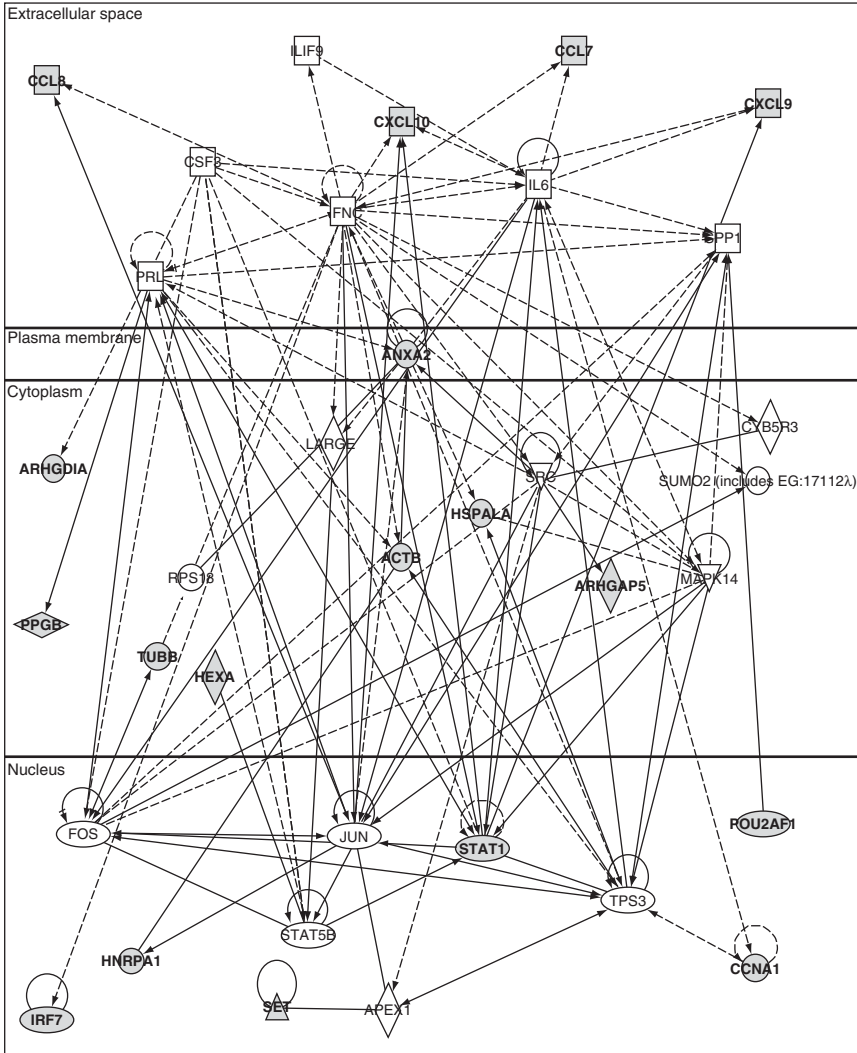
Another study examined the effects of Tat expression in human astrocytes, as a means to examine processes important in HIV-1-associated dementia (Pocernich *et al.*, 2005). This study also observed downregulation of  $\beta$ -tubulin and components of the Rac/RHO-GDI complex. Other proteins downregulated 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'Keefe *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; NF $\kappa$ B, 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 NF $\kappa$ B 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 lipodystrophy (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  $\delta$ -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 NF $\kappa$ B 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  $\mu$ RPLC-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 anti-viral 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

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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  $\gamma$ -chain knockout) are irradiated and then implanted with human CD34<sup>+</sup> 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

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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.

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

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## **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

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<sup>+</sup> 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

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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

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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

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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<sup>+</sup> 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

<i>Subtype</i>	<i>Source</i>	<i>Strain</i>	<i>Isolate/form</i>	<i>Disease potential</i>	<i>Other comments</i>	
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-smDeltaB670	Uncloned	High, AIDS	RP in combination with 17E-Fr	
SIV-sm	TNPRC	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<sup>+</sup> T cells at mucosal sites (Brenchley *et al.*, 2004; Veazey *et al.*, 1998, 2000).

## V. Pathogenesis of SIV in Macaques

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### 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<sup>+</sup> 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.

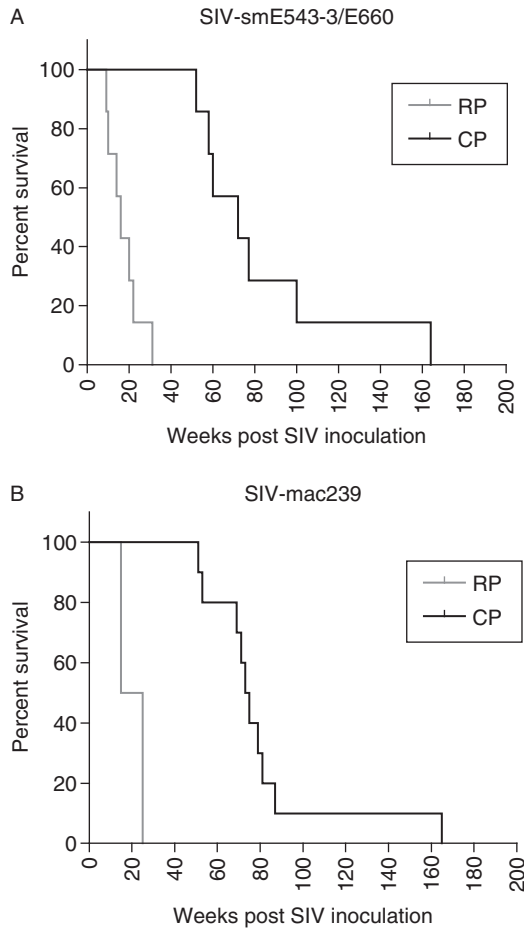
The pathogenesis of SIV-sm/mac infection in macaques appears to be remarkably similar to human AIDS. Both show a period of intense viremia during the first four weeks of infection (primary) with rapid dissemination of virus to lymphoid and mucosal sites. The primary phase of infection is associated with a sharp decline in memory CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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/ $\mu$ l. 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-smPBj 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<sup>+</sup> T cells in blood and mucosal sites, (2) plasma viral load, and (3) the degree of immune activation.

### I. CD4<sup>+</sup> T Cells in Blood and Mucosal Sites

The initial predictors of absolute CD4<sup>+</sup> T cells in the circulation or the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> 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<sup>+</sup> T-cell subsets is a valuable predictor of disease progression. Veazey *et al.* (1998) initially demonstrated selective depletion of mucosal CD4<sup>+</sup> 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<sup>+</sup> T cells subsequently revealed that mucosal CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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

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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<sup>+</sup> T Cells

Surprisingly considering the rapidity of the disease course in these animals, the total numbers of CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells is the mechanism underlying immune failure in RP macaques. Naive CD4<sup>+</sup> T cells are relatively preserved terminally in RP macaques; this contrasts with a generalized loss of both memory and naive CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> T cells and the disruption to germinal centers are likely to contribute to the failure of these animals to maintain SIV-specific immune responses.

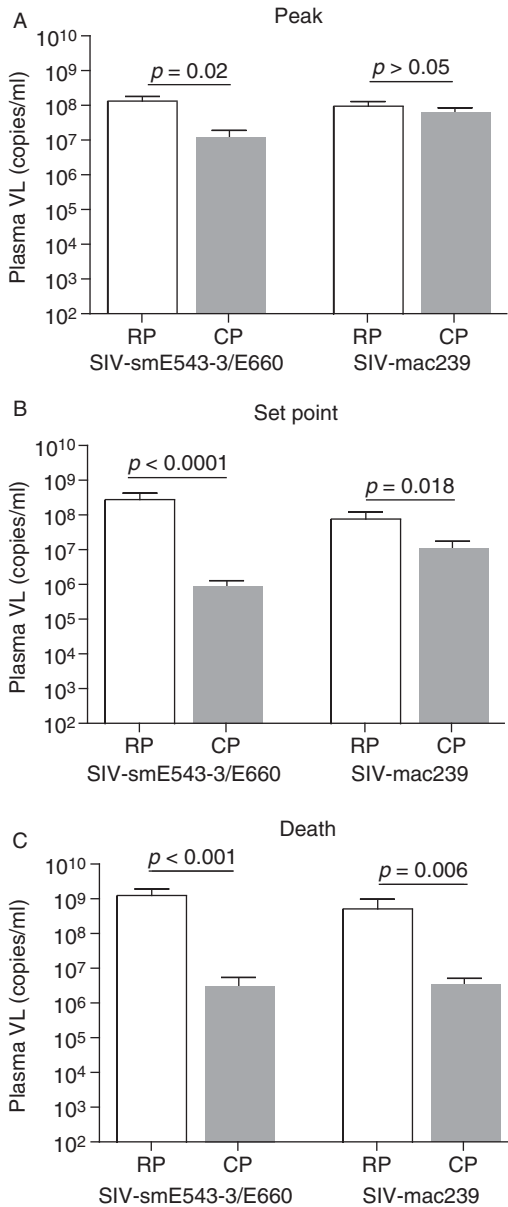
## B. Virological Features

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.

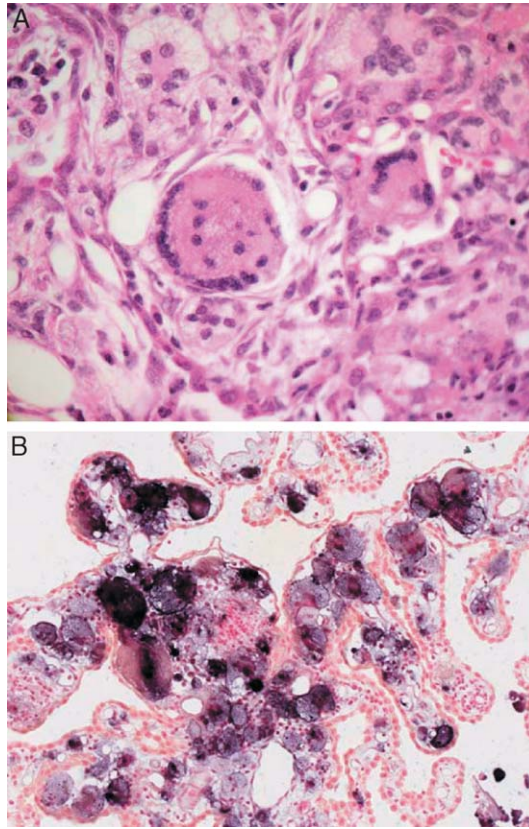
## C. Pathological Features

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 as 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.



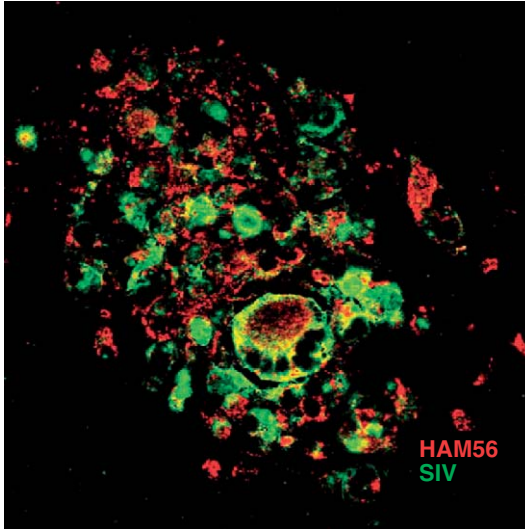
**FIGURE 2** Plasma viremia of macaques infected with SIV-smE543-3/E660 or SIV-mac239 at: (A) Peak plasma viral load (VL), (B) Plasma viral load at set point, and (C) Plasma viral load at death. RP macaques are shown as white bars and CP macaques are shown as gray bars. The error bars represent the standard 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<sup>+</sup> 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 (Sauermaun *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<sup>+</sup> 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<sup>+</sup> T cells, demonstrating that CTL plays an important role in pathogenesis (Jin *et al.*, 1999; Schmitz *et al.*, 1999). In fact, when CD8<sup>+</sup> 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

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### 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-mac239 $\Delta$ nef variant remaining healthy for years (Alexander *et al.*, 2003). Furthermore,  $\Delta$ nef 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.

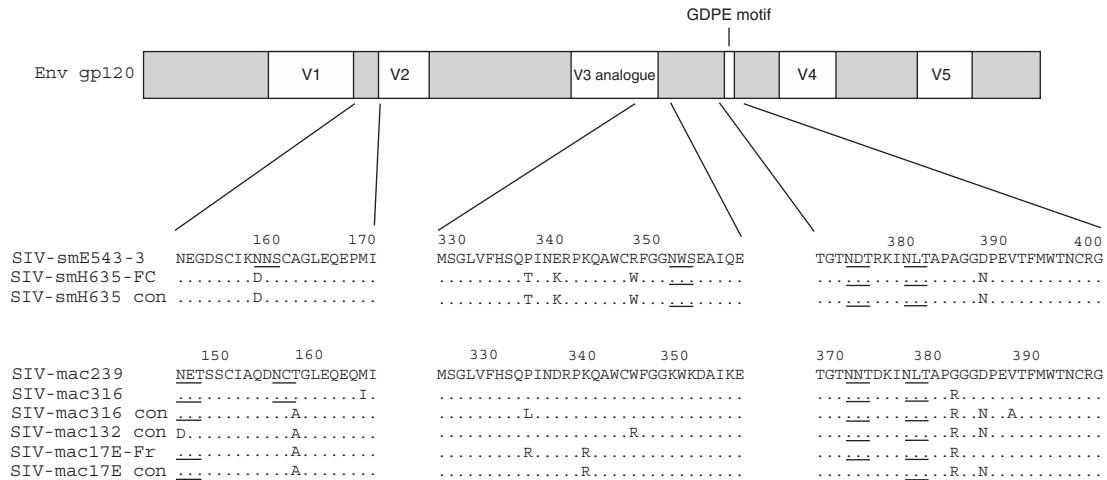
## B. Evolution of SIV in RPs

### 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 SIV-mac316, 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<sup>+</sup> 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

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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<sup>+</sup> 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<sup>+</sup> 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.

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# Nonprimate Models of HIV-1 Infection and Pathogenesis

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## I. Chapter Overview

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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

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

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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<sup>-/-</sup>  $\gamma$ c<sup>-/-</sup> 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

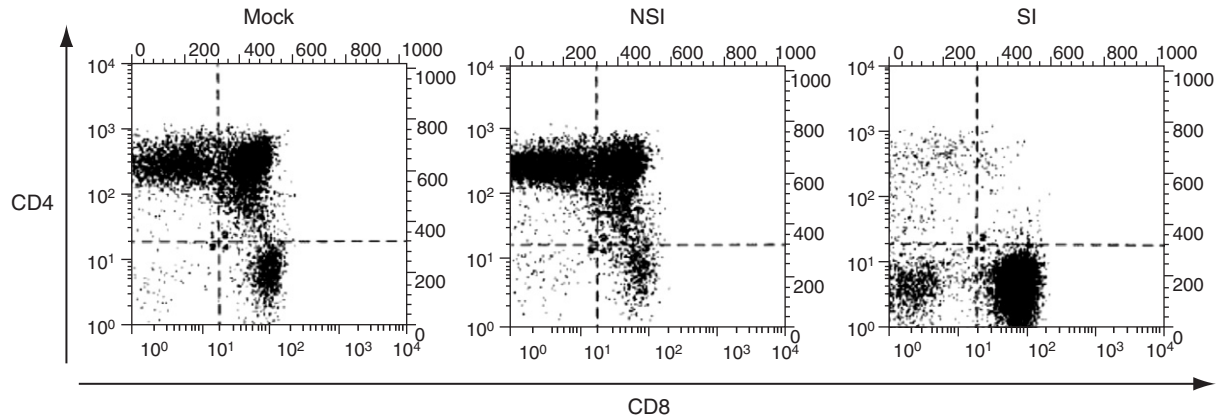
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The SCID-hu Thy/Liv mouse is created by coimplantation of human fetal thymus and liver under the kidney capsule of an SCID mouse, resulting in a conjoint Thy/Liv organ. Human fetal liver is included to provide a source of human hematopoietic progenitor 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 hemato lymphoid 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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 V $\beta$  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  $\alpha/\beta$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 I** 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<sup>+</sup> 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  $\beta$ -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-1 $\alpha$ , MIP-1 $\beta$ , 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<sup>+</sup> T-cell depletion accelerates probably because CXCR4 is present on a larger subset of T cells, although the mechanisms of CD4<sup>+</sup> 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<sup>+</sup> 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

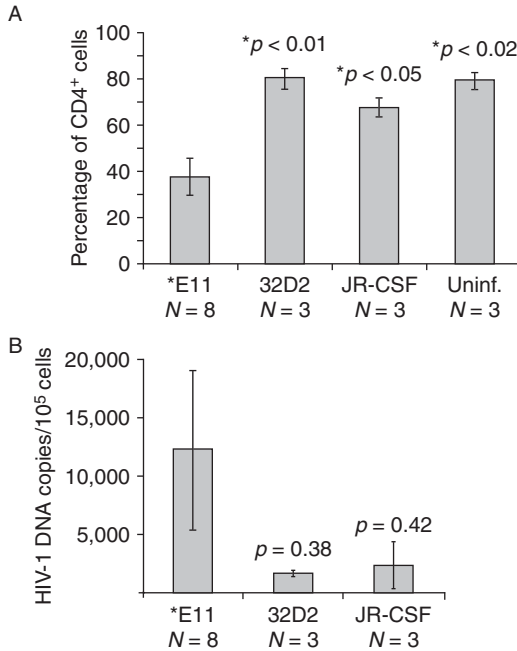
from virus isolated from the laboratory worker (HXB2/LW). HXB2/LW clones containing the *env* gene derived from the laboratory worker replicated efficiently in SCID-hu mice. The enhanced replication was attributed to six unique mutations in the third variable region of gp120 (V3 loop) of the recombinant 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<sup>+</sup> 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

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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<sup>+</sup> or CD8<sup>-</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells in all infected grafts at eight weeks postinfection. (B) HIV-1 strong-stop DNA detected per 10<sup>5</sup> 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  $\beta$ -globin copies. The number of HIV-1 DNA copies was normalized to cell number by measuring the number of  $\beta$ -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

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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<sup>+</sup> T cells, however, were not maintained at a

normal ratio to CD8<sup>+</sup> T cells, which was detrimental to studies of HIV-1. To enhance CD4<sup>+</sup> T-cell engraftment, NOD-SCID  $\beta_2$ -microglobulin-null (NOD-SCID- $\beta_2m^{\text{null}}$ ) mice were subsequently created by the same group (Christianson *et al.*, 1997). NOD-SCID and NOD-SCID- $\beta_2m^{\text{null}}$  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<sup>-/-</sup> $\gamma$ C<sup>-/-</sup> and NOD-SCID $\gamma$ C<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> and NOD-Rag<sup>-/-</sup> mice by several groups has eliminated this problem (Ito *et al.*, 2002; Shultz *et al.*, 2000; Traggiai *et al.*, 2004). The Rag<sup>-/-</sup> 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 ( $\gamma$ c), 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<sup>-/-</sup> mutation into Balb/C Rag<sup>-/-</sup> 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<sup>+</sup> HSC into adult or newborn mice (Ishikawa *et al.*, 2005; Ito *et al.*, 2002; Shultz *et al.*, 2005; Traggiai *et al.*, 2004). Recipient Rag<sup>-/-</sup>  $\gamma$ c<sup>-/-</sup> or NOD-SCID  $\gamma$ c<sup>-/-</sup> 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<sup>-/-</sup>  $\gamma$ c<sup>-/-</sup> or NOD-SCID  $\gamma$ c<sup>-/-</sup> 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<sup>+</sup> T-cell depletion in peripheral blood, spleen, and thymus, and the appearance of anti-HIV-1 antibodies. This new Rag<sup>-/-</sup>  $\gamma$ c<sup>-/-</sup> or NOD-SCID  $\gamma$ c<sup>-/-</sup> mouse model of HIV-1 infection is a very promising development.

## VII. Humanized Immune Competent Mice and Rats

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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 cyclin-T1 was also beneficial (Keppler *et al.*, 2001). Coexpression of human CD4 and CCR5 in Tg rats leads to expression on rat CD4<sup>+</sup> T cells, macrophages, and microglia but not on rat CD8<sup>+</sup> 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

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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  $\Delta$ *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- $\alpha$  (TNF- $\alpha$ ) was an important mediator of disease in NL4-3  $\Delta$ *gag-pol* Tg mice and that hCG reversed the symptoms by lowering TNF- $\alpha$  levels (De *et al.*, 2002). Moreover, a similar decrease in HIV-1 gene expression and amelioration of symptoms resulted from administration of anti-TNF- $\alpha$  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<sup>+</sup> 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<sup>+</sup> cells compared to mice expressing JR-CSF alone and also exhibited depletion of CD4<sup>+</sup> 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<sup>nef</sup>), but not in HIV-1 Tg mice that lacked functional *nef* (Weng *et al.*, 2004). Peripheral CD4<sup>+</sup> T cells isolated from CD4C/HIV<sup>nef</sup> Tg mice exhibit an activated/memory phenotype with higher expression of CD25 and CD69 on CD4<sup>+</sup> 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<sup>+</sup> 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 astrogliosis, 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

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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<sup>+</sup> 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

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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.

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# Perspectives for a Protective HIV-1 Vaccine

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## I. Chapter Overview

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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);

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

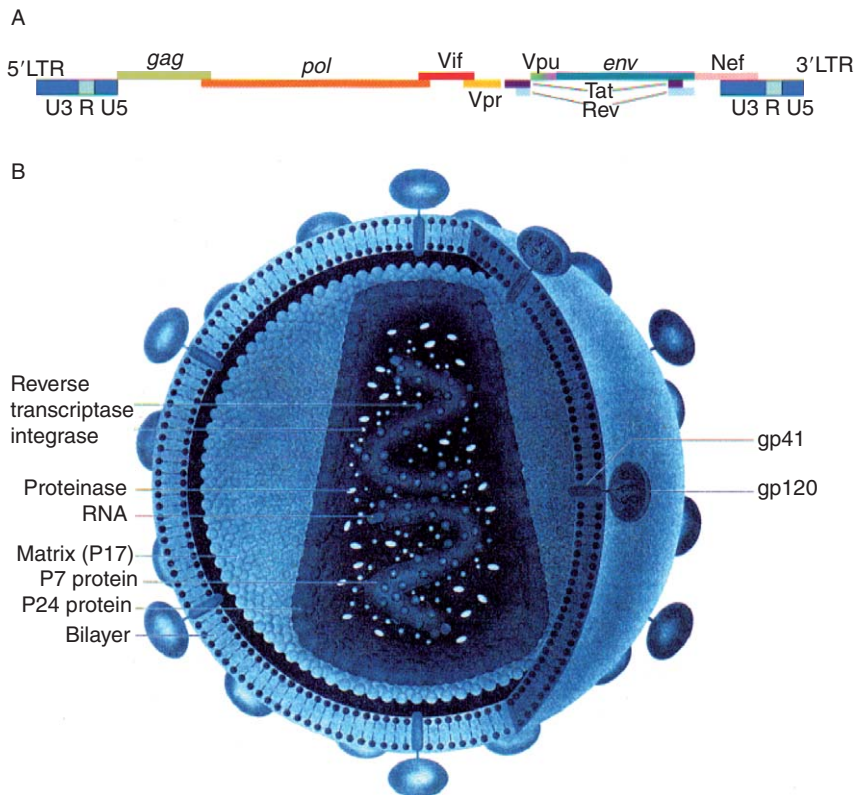
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The continuous spreading of the HIV-1 infection worldwide is a major health problem with an estimated 39.4 million of subjects infected at the end of 2004 (WHO/UNAIDS report). This burden is increasing at the annual rate of 5 million new infections, 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



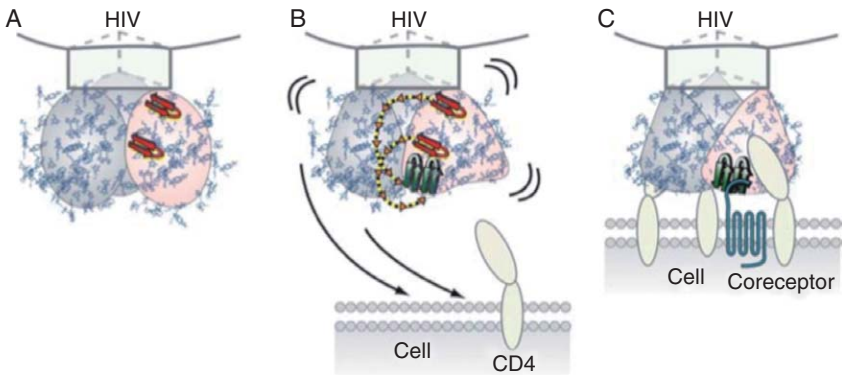
**FIGURE 1** (A) Schematic representation of the viral genome containing replication genes (*pol*, *vif*, *nef*, *tat*, *rev*, *vpu*, *vpr*) and assembly genes (*gag*, *env*); (B) HIV-1 physical structure. The glycosylated envelope surrounds the viral matrix that contains the viral genome and enzymes 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<sup>+</sup>CCR5R<sup>+</sup> 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  $\beta$ -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



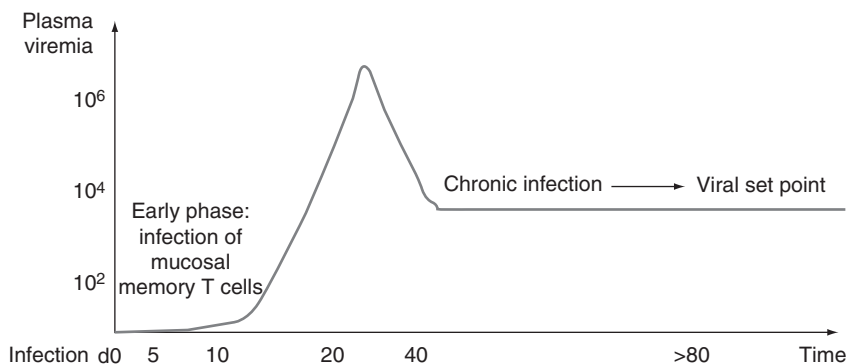
**FIGURE 2** Changes in the structure of the HIV envelope on binding to target cells. (A) The heavily glycosylated envelope comprises gp120 and gp41 as a trimer. This structure was solved by [Chen \*et al.\* \(2005\)](#) for SIV. (B) When the envelope binds to CD4, the gp120 undergoes a conformational change that forms and exposes the binding site for the coreceptor CCR5 or CXCR4 (shown as red  $\beta$  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 initial 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- $\gamma$  (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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T-cell response is also detectable during the primary infection, and may provide T help to CD8<sup>+</sup> effector T cells; however, this function is undermined by the susceptibility of CD4<sup>+</sup>CCR5<sup>+</sup> 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- $\gamma$ , IL-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , 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<sup>+</sup>CCR5<sup>+</sup> 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

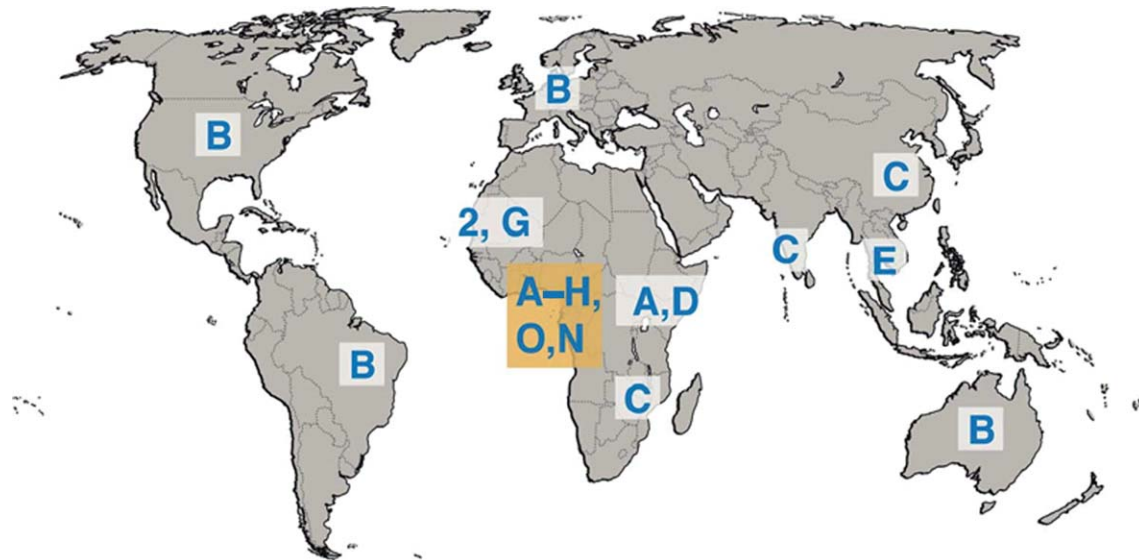
highly HIV-1-exposed and -uninfected people have been identified that show variable levels of HIV-1-specific CD8<sup>+</sup> T-cell and/or CD4<sup>+</sup> T-cell responses (Clerici *et al.*, 1994; Goh *et al.*, 1999; Hladik *et al.*, 2003; Rowland-Jones *et al.*, 1995, 1998). Consistently, in these subjects 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/ $\Delta$ 32 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<sup>+</sup> T-cell levels, possibly indicating that the  $\Delta$ *nef* HIV was not pathogenic. Three of the seven members of SBBC were long-term non-progressors (LTNP) with near normal CD4<sup>+</sup> 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<sub>p24</sub>. Two recipients and the original donor showed a significant decline of CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells as compared with the 1995 sample. Indeed, while the 1995 viral strain was restricted to CCR5<sup>+</sup> cells, the 1999 isolate could efficiently infect both CCR5<sup>+</sup> and CXCR4<sup>+</sup> 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 SIVmac8 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, SIVmac293 $\Delta$ *nef* (SIV $\Delta$ *nef*) containing an inactivating deletion of the *nef* gene, and SIVmac293 $\Delta$ 3 (SIV $\Delta$ 3), 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 SIV $\Delta$ *nef* or SIV $\Delta$ 3 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 SIV $\Delta$ 3, SIVmac293 $\Delta$ 3X, or SIVmac293 $\Delta$ 4 (SIV $\Delta$ 4) 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<sup>+</sup> 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 SIV $\Delta$ 3 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 SIV $\Delta$ 3 and challenged intravenously with SHIV89.6PD were protected from CD4<sup>+</sup> 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 SIV $\Delta$ 3 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-1<sub>NL4-3</sub> 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- $\gamma$  expression), and live-attenuated SHIV-4 (deleted *vpu* and *nef*) and SHIV<sub>ppc</sub> (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- $\gamma$ ) 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<sup>+</sup> 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<sup>a</sup>

<i>Delivery</i>	<i>Clade</i>	<i>Immunogen</i>	<i>Phase</i>
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

<sup>a</sup>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, *Venezuelan 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<sup>+</sup> 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<sup>+</sup> T-cell responses (McShane *et al.*, 2004).
5. Recombinant, replication defective adenovirus-5 provides a substantial and sustained CD8<sup>+</sup> 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**

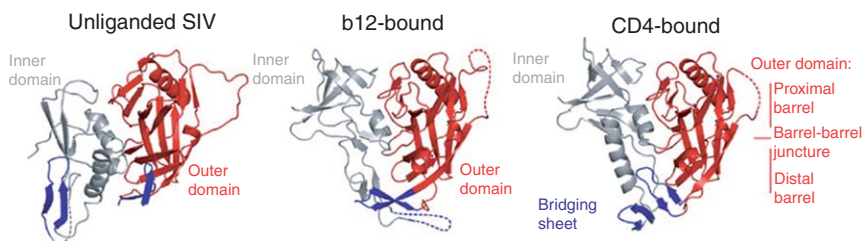
Several studies have shown that neutralizing antibodies with broad specificity can protect from the onset of HIV-1 infection if present at a threshold concentration at the time of the viral inoculum. Indeed, passive administration of broadly neutralizing monoclonal HIV-1 envelope-specific antibodies 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 features 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  $\beta$ -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

<i>Antibody</i>	<i>Epitope specificity</i>	<i>Features</i>	<i>Reference</i>
b12	CD4-binding site	Extended CDR3 region	Saphire <i>et al.</i> , 2001
X5	CD4 induced (coreceptor- binding site)	Effective as Fab fragment only	Labrijn <i>et al.</i> , 2003
2G12	Complex mannose	Antibody has VH domain swap	Calarese <i>et al.</i> , 2003
2F5	Proximal domain of gp41	IgG3 with long CDR3; cross-reacts with cardiolipin	Haynes <i>et al.</i> , 2005; Zwick <i>et al.</i> , 2004
4E10	Conserved membrane	IgG3 with long CDR3; proximal domain of gp41 cross-reacts with cardiolipin	Cardoso <i>et al.</i> , 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).

### ***I. 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 earlier, 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<sup>+</sup> 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

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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.

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

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## **I. Chapter Overview**

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*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<sup>+</sup> 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

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

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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<sup>+</sup> 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 (Scarlati *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 (Scarlati *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 possess 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells to 43% at counts of <200 and was also related to the percentage of CD8<sup>+</sup> cells with the lowest risk (12%)

when the CD4<sup>+</sup> 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/ $\mu$ l (Fang *et al.*, 1995) and 75% of the women transmitting with HIV-1 RNA levels over 50,000 copies/ $\mu$ l (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

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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

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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<sup>+</sup> 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

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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.

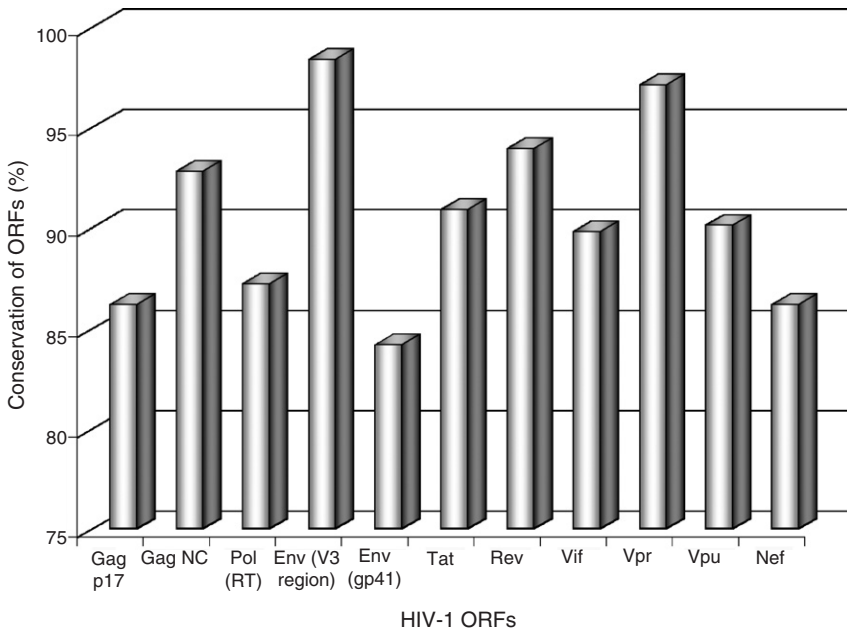
The *env* gp120 binds to CD4 receptors and coreceptors located on the plasma membrane of CD4<sup>+</sup> T lymphocytes, monocytes, macrophages, and dendritic cell (Weiss, 1993). Comparison of the sequences of *env* genes from numerous HIV-1 isolates reveals a pattern of five variable regions (V1–V5) 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.

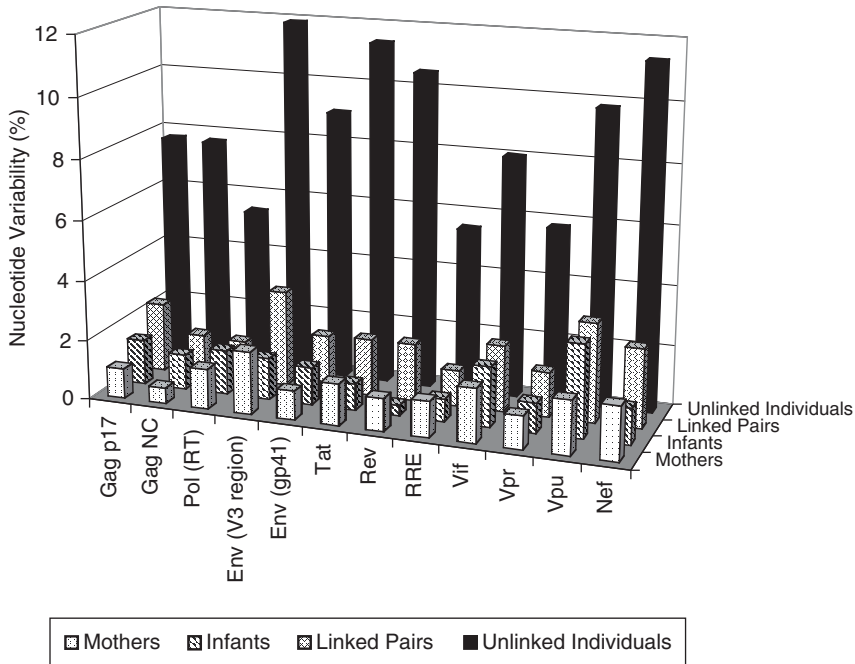
The regulatory genes *tat* and *rev* and accessory genes *vif*, *vpr*, *vpu*, and *nef* are required for viral replication and pathogenesis. In addition, the accessory genes *vif* and *vpr* are found to be highly conserved and functional during natural infection (Goh *et al.*, 1998; Sova *et al.*, 1995), 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

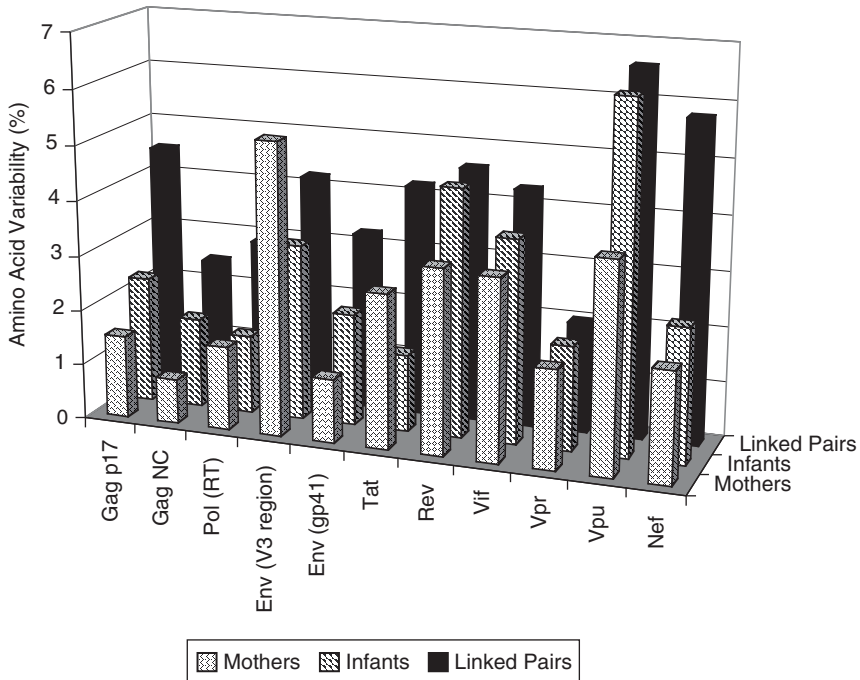


**FIGURE 1** Frequency of conservation of intact HIV-1 ORFs, including Gag p17 and NC, Pol RT, Env (V3) and gp41, Tat, Rev, Vif, Vpr, Vpu, and Nef from deduced amino acid sequences from mother–infant isolates following vertical transmission. The frequency of conservation of ORFs is expressed as percentages 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* p17 and 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

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The functional domains required for the activity of HIV-1 genes including Env V3 region and gp41, Gag p17 and 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 (Miyachi *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<sup>2+</sup>-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  $\alpha$ -helix H and  $\alpha$ -helix I (Jacobso-Molina *et al.*, 1993; Kohlstaedt *et al.*, 1992), were 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; Jacobso-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  $\alpha$ -helices, comprising of residues 9–24 and 34–62 (Watts *et al.*, 1998). The two  $\alpha$ -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 phosphorylation, and cyclic GMP-dependent protein kinase phosphorylation 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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 (~27 kDa) (Ahmad and Venkatesan, 1988) and features a structurally flexible N-terminal arm of ~70 residues and a conserved, highly structured core domain of ~120 residues (residues ~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<sup>2</sup>) 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<sup>57</sup>, L<sup>58</sup>, and E<sup>59</sup> 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<sup>160</sup>xxxLL<sup>165</sup>) 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<sup>177</sup>) was conserved in all mother–infant pairs' sequences, since the E → G<sup>177</sup> 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  $\alpha$ H1 (Mangasarian *et al.*, 1999), including the indispensable M<sup>20</sup> 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<sup>69</sup>xxPxxPxxP<sup>78</sup> 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<sup>69</sup>xxPxxPxxP<sup>78</sup> motif were highly conserved in our mother–infant pairs' *nef* sequences (Hahn *et al.*, 2003).

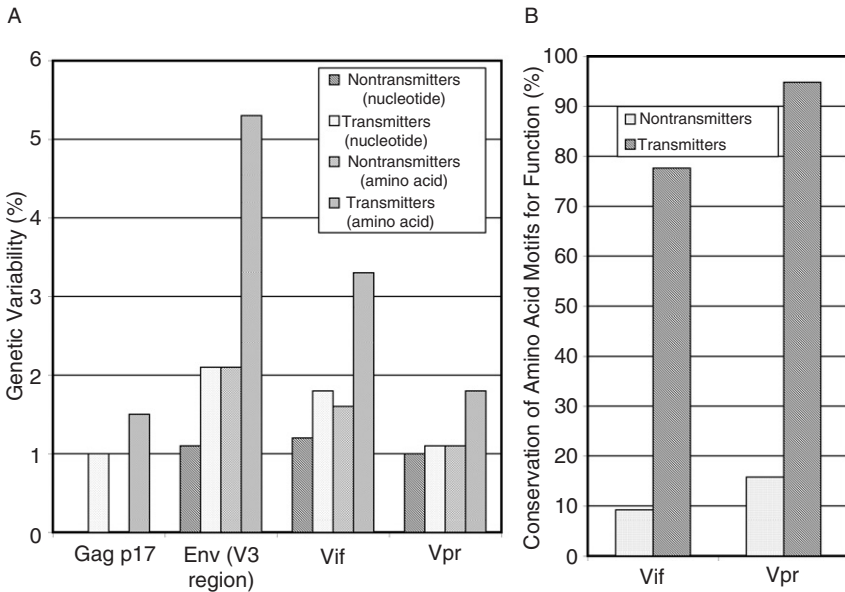
## **XI. Properties of HIV-1 Associated with Lack of Vertical Transmission**

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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 asparagine 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](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](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](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, GNFLQSRPEPTAPPE, is located at amino acid positions 70–84 and is recognized by several HLA types ([http://www.hiv.lanl.gov/content/immunology/ctl\\_search](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](http://www.hiv.lanl.gov/content/immunology/ctl_search)). This epitope is recognized by HLA-B60 and is positioned within the (LXX)<sub>4</sub> 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](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 epitope, TVLDVGDAY, between amino acid positions 107 and 115 ([http://www.hiv.lanl.gov/content/immunology/ctl\\_search](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](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  $\alpha$ -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**

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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<sup>+</sup> cells, drastic reduction in cortical CD4/CD8 double



positive cells (Rubinstein *et al.*, 1983; Stanley *et al.*, 1993), and an increased percentage of CD8<sup>+</sup> 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-1 $\beta$ , IL-2, IL-6, and interferon- $\gamma$  in lymph nodes, and decreased production of IL-2, IL-4, and interferon- $\gamma$  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<sup>+</sup> 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



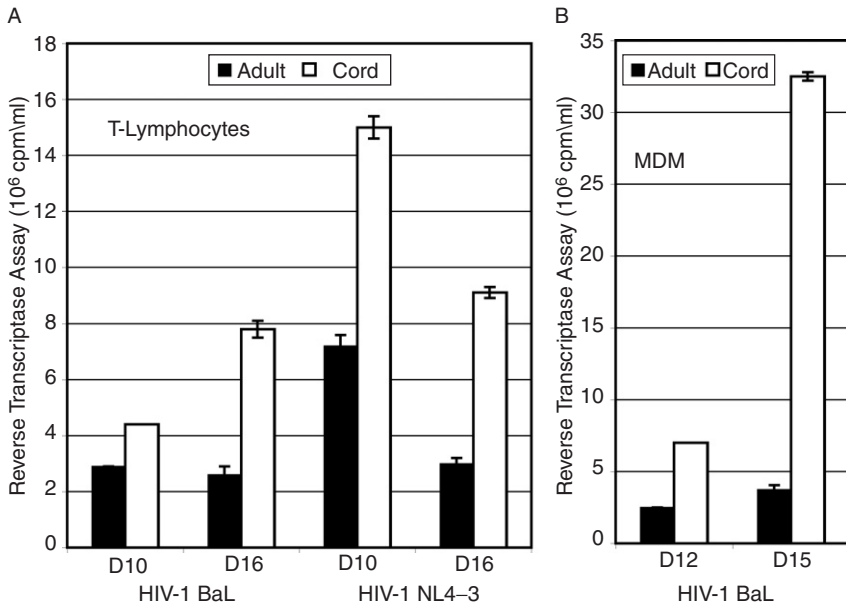
over time (Zhang *et al.*, 2006). However, there was no significant difference in the growth properties of viruses from rapid or slow progressors as well as humoral immune response was not predictive of disease progression (Zhang *et al.*, 2006).

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<sup>+</sup>/CCR5<sup>+</sup> positive T cells may also play an important role in pathogenesis of HIV disease. The majority of HIV-1-infected CD4<sup>+</sup> T cells in the blood of most infected infants and children have the memory (CD45RO<sup>+</sup>) phenotype, despite the relative scarcity of these cells (Sleasman *et al.*, 1996), because the CD4<sup>+</sup> T cells of infants are enriched in cells of the naive (CD45RA<sup>high</sup>) phenotype that express higher levels of CXCR4 (Mo *et al.*, 1998). Infection of naive (CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells is associated with a rapid decline in CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cells in a thymic organ culture (Clark *et al.*, 1997). Our preliminary results suggest that the development of CD4<sup>+</sup> 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<sup>+</sup> 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.

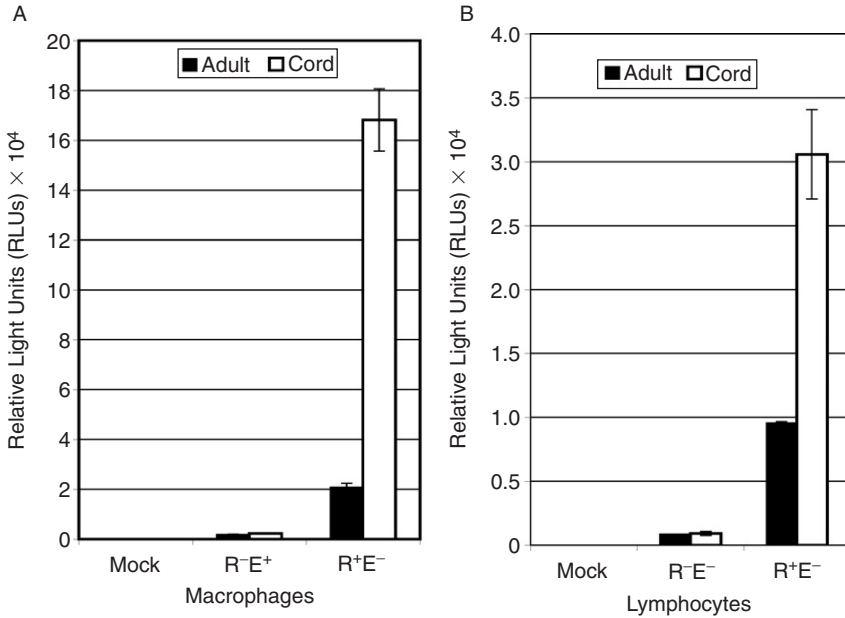
We have compared HIV-1 replication kinetics between neonatal and adult blood mononuclear cells and determined the mechanisms of HIV-1 replication in these cell types. We have used cord blood in place of neonatal blood because, like neonatal blood, it has more CD45RA<sup>+</sup> T cells and less CD45RO<sup>+</sup> 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 ( $1 \times 10^6$ ) and MDM ( $0.5 \times 10^6$ ) from seven different donors with HIV-1. Equal number of lymphocytes and MDM isolated were infected with equal amount ( $5\text{--}15 \times 10^4$  RT counts per minute) of HIV-1<sub>BaL</sub> and HIV-1<sub>NL4-3</sub> (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-1<sub>BaL</sub> replication in cord and adult blood T lymphocytes and MDM are shown (Sundaravaradan *et al.*, 2006). The data clearly demonstrates that HIV-1<sub>BaL</sub> 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-1<sub>BaL</sub> replicated ninefold better in cord MDM compared with adult MDM (Fig. 5B). We also found that HIV-1<sub>NL4-3</sub> 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  $\pm$  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<sup>-</sup> (R<sup>+</sup>/R<sup>-</sup>) that measures transcriptional activity of HIV-1 LTR (Connor *et al.*, 1995). Equal amounts of ( $1 \times 10^5$ ) RT counts of the NL-Luc-E<sup>-</sup> (R<sup>+</sup>/R<sup>-</sup>) 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<sup>-</sup> (R<sup>+</sup>/R<sup>-</sup>)] 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)  $\pm$  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**

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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.

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# The Viral Etiology of AIDS-Associated Malignancies

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## I. Chapter Overview

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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  $\gamma$ -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

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

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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 median 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

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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 coinfecting 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 mantle 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  $\gamma$ -herpesvirus subfamily, which can be further subdivided into two subgroups, gamma-1 or lymphocryptovirus, 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  $\sim$ 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  $\gamma$ -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  $\gamma$ -herpesvirus, EBV, which belongs to the lymphocryptovirus genus. A feature of  $\gamma$ -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  $\gamma$ -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

<i>Viral gene</i>	<i>Function</i>
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- $\kappa$ B
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-1 $\alpha$ . They induce signal transduction and enhance angiogenesis
vFLIP (viral FLICE-inhibitory protein or ORF71)	Homologue of cellular FLIP, it activates NF- $\kappa$ B 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- $\kappa$ B 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 cytohesin-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 p38 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  $\alpha$  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-1 $\alpha$  (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- $\kappa$ B 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  $\beta$ -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

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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<sup>+</sup> 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, non-polyadenylated 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 I (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- $\kappa$ B 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- $\kappa$ B 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 Antigen I (EBNA-I)**

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 EB1/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-J $\kappa$  (also called RBP-J, CBF1, KBF2, or CSL). EBNA-2 is essential for EBV-induced immortalization of B lymphocytes and complex formation with RBP-J $\kappa$  is crucial for such activity. RBP-J $\kappa$  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-J $\kappa$  and modulates the activity of target promoters. Although Notch1 IC and ENBA-2 share the ability to transactivate genes by interacting with RBP-J $\kappa$ , 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

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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 tumorigenicity 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-J $\kappa$  (Liang *et al.*, 2002), a key cellular target of the EBV latent transforming program (Zimmer-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

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### 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 Tying, 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.

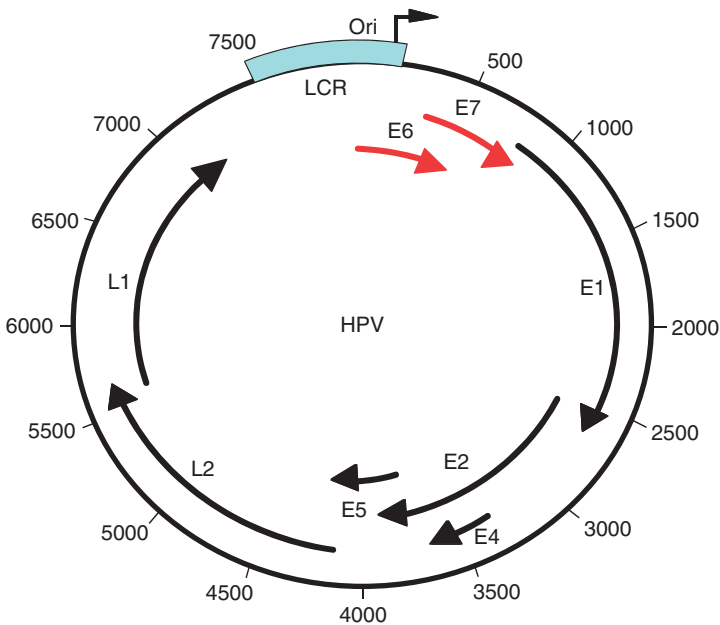
## VII. HPV—The Causative Agent

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### 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  $\alpha$  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).



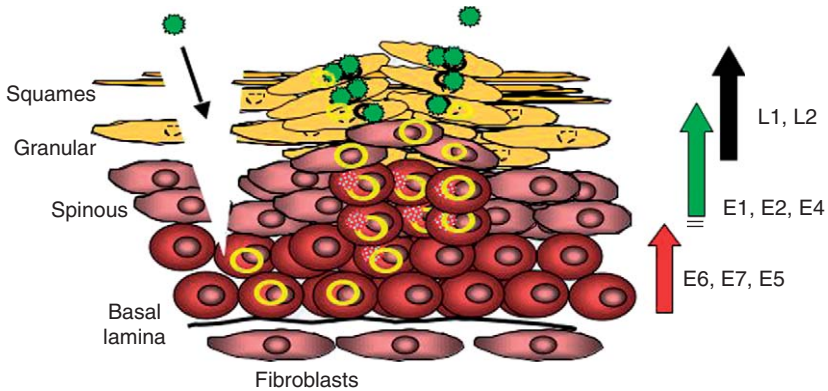
**FIGURE I** The HPV genome is a 7.9 kb double-stranded circular genome. The genome is controlled by a single keratin-dependent promoter element; the long control region (LCR; in blue). At the 3' end of the LCR is the origin of replication (*nucleotide position 1*). E6, E7 (red), and E5 are viral oncogenes; 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

<i>Designation</i>	<i>Function</i>
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 $\alpha$ . Significant structural homology to SV40 large T antigen (Clertant and Seif, 1984; Mansky <i>et al.</i> , 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) <sub>4</sub> CGGT 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 cyokeratin 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 $\beta$ 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.

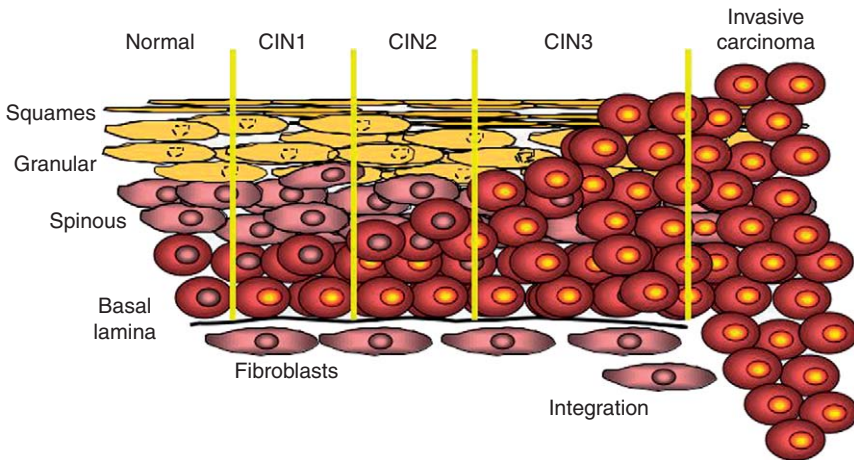


Infection by high-risk HPVs, especially HPV16, can induce warts; low-grade dysplasias, CIN, CIN 1 and CIN 2 designations are reversible forms of precancerous lesions (Fig. 3). Integration of the HPV genome can lead to an increase of expression of E6 and E7 resulting in progression to CIN 3, which is 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.

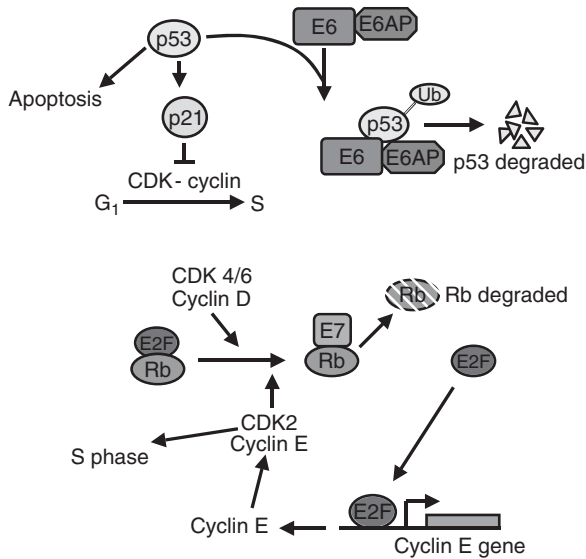
### E. The Mechanism of HPV-Induced Transformation and Cancer Progression

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

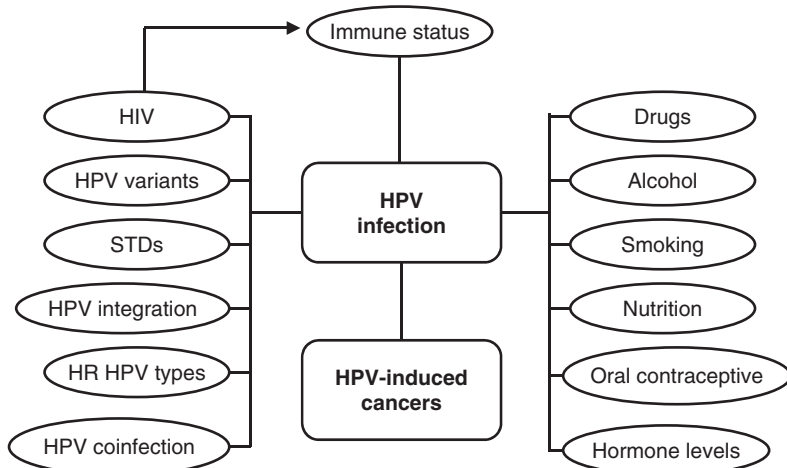


**FIGURE 4** Diagram of the role of E6 and E7 in dysregulation of the cell cycle. Expression of E6 leads to recruitment of E6AP (a ubiquitin ligase). This complex causes degradation of p53, which then inhibits the p21-dependent block of the G<sub>1</sub> to S transition. Similarly, E7 binding to Rb displaces 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

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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.

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