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PLATINUM AND OTHER METAL COORDINATION COMPOUNDS IN CANCER CHEMOTHERAPY

Proceedings of the Fifth International Symposium on Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy Abano, Padua, ITALY - June 29–July 2, 1987

edited by

Marino Nicolini
University of Padua
Padua, Italy

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"Pone ergo mentem tuam super salem
nec cogites de aliis.
Nam in ipsa sola occultatur scientia
et arcanum praecipuum,
et secretissimum omnium antiquorum Philosophorum".

(Rosarium Philosophorum)

"Address, thus your mind to salt, and do not take heed of other compounds. For in it only are hidden science and the foremost and deepest secret of all ancient philosophers".

(Rosarium Philosophorum)

Preface

This book records the proceedings of the Fifth International Symposium on Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy which was held at Abano (Padua, Italy) on June 29-July 2, 1987. Since the Fourth such Symposium, held in Burlington, Vermont, in 1983 several advances has occurred in the chemistry, biochemistry, pharmacology, toxicology and clinical use of platinum and other metal complexes to demand a new conference on the field.

This volume includes the manuscripts of Plenary and Invited Speakers who were charged to review recent developments and point to some new promising areas of research. Also included are full papers based on poster contributions which were deemed worth of publication by an international panel of referees drawn from the Scientific Advisory Board of the Symposium. Out of the 157 posters presented at the Conference and discussed in Microsymposia, most described work still in progress and were not submitted by their authors for this volume and a few were rejected. All the conference sections devoted to primary aspects of the use of platinum and

other metals in cancer chemotherapy are represented in this volume. The questions raised and answers given during the discussions following each lecture through active participation by the attendees are not recorded, unfortunately, but were certainly a major feature of the success of the Conference which gathered some 300 conveners.

I wish to record here my indebtedness to B. Rosenberg, Honorary President, M.P. Hacker and S.J. Lippard, Co-chairmen, the Scientific Advisory Board, and especially to P. Uguagliati, the Secretary, and to all those of my Colleagues of the Organizing Committee whose help was essential for the smooth running of the Symposium.

It is my pleasure also to record our gratitude to the sponsors who gave their financial support, as listed at the beginning of the volume.

Marino Nicolini

University of Padua - August 10, 1987

At the Opening Ceremony preceding the Conference professor B. Rosenberg was awarded the Galilei Gold Medal of the University of Padua in recognition of his pioneering work in platinum chemotherapy of cancer.

He then gave the conveners a first-hand account of the origin and early developments of his discovery of cis-platin.

At the end of the Conference of three best posters as judged by an international panel of referees were awarded the Galilei Silver Medal in recognition of their authors efforts toward a clear but concise presentation of results. Awardees were Dr. A.C. Begg, the Netherlands Cancer Institute (C 2), Dr. K.A. Skov, B. Columbia Cancer Res Center (E 40), and Dr. H. Schönenberg, Lehrstuhl für Pharmazeutische Chemie II, Universität Regensburg (C 26).

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Contributors

Y. ADACHI
Shionogi Research Laboratories
Shionogi & Co.
Fukushima - Ku
Osaka 553
Japan

H. ADOMAT
Cancer Research Centre
601 West 10th Avenue
Vancouver, B.C. Y5Z 1L3
Canada

SURINDER K. AGGARWAL
Dept. of Zoology and Biological Sciences
Michigan State University
East Lansing, MI 48823
USA

K. AKAMATSU
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

G.M. ALEKSEYEVA
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

ENZO ALESSIO
Dip. di Scienze Chimiche
Università di Trieste
P.le Europa, 1
34127 - Trieste
Italy

DOMENICO AMOROSO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

ALAN R. AMUNDSEN
Engelhard Corporation
Menlo Park CN-28
Edison, NJ 08818
USA

PAUL A. ANDREWS
Dept. of Medicine and Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

PETER J. ANDRULIS, Jr.
Andrulis Research Corporation
11800 Baltimore Ave.
Beltsville, MD 20705
USA

PETER ANDRULIS, III
Andrulis Research Corporation
11800 Baltimore Ave.
Beltsville, MD 20705
USA

K. AONO
Shionogi Research Laboratories
Shionogi & Co.
Fukushima - Ku
Osaka 553
Japan

TREVOR G. APPLETON
University of Queensland
Dept. of Chemistry
Brisbane 4067
Australia

JORMA ARPALAHTI
Institut für Anorganische und
Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

PAOLA ARSLAN
Istituto di Patologia Generale
Università di Padova
Via Loredan, 16
35131 - Padova
Italy

W. ATTIA
Dept. of Physics
Suez Canal University
Ismailia
Egypt

E.G. BAKALBASSIS
Dept. of General and Inorganic
Chemistry
University of Thessaloniki
54006 - Thessaloniki
Greece

H. BARTELINK
The Netherlands Cancer Institute
Dept. Exp. Radiotherapy
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

A. BARZI
Istituto di Farmacologia
Università di Perugia
Via del Giochetto
06100 - Perugia
Italy

ADRIAN C. BEGG
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

M. BELTRAME
Istituto di Patologia Generale
Università di Padova
Via Loredan, 16
35131 - Padova
Italy

F. BERENDS
Medical Biological
Laboratory TNO
2280 AA Rijswijk
The Netherlands

M.R. BERGER
Institut für Toxikologie und
Chemotherapie am Deutschen
Krebsforschungszentrum
Im Neuenheimer Feld 280
D-6900 Heidelberg
FRG

S.J. BERNERS PRICE
Dept. of Chemistry
Birkbeck College
University of London
Malet Street
London, W C1E 7 HX
UK

FRITJOF BERNGES
Institut für Biophysik und physikalische
Biochemie der Universität
Universitätsstrasse, 31
D-8400 Regensburg
FRG

J. BERNHEIM
Faculty of Medicine and Pharmacy
Free University of Brussels
Laarbeeklaan 103/E
B-1090 Brussels
Belgium

G. BERTELLI
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16136 - Genova
Italy

TITO BERTI
Dip. di Farmacologia
Università di Padova
Largo Meneghetti, 2
35131 - Padova
Italy

HELGE BISCHOFF
Institut für Toxicologie und
Chemotherapie am Deutschen
Krebsforschungszentrum
In Neuenheimer Feld 280
D-6900 Heidelberg
FRG

JAMIE BISWAS
Andrulis Research Corporation
11800 Baltimore Ave.
Beltsville, MD 20705
USA

P. BITHA
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

R. BLECHOVÁ
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

SILVIA BÖHM
Istituto Nazionale per lo Studio
e la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

M. BOHUMINSKÁ
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

GIAN MARIA BONORA
Centro di studi sui Biopolimeri, C.N.R.
Via Marzolo, 1
35131 - Padova
Italy

RICHARD F. BORCH
Dept. of Pharmacology and
Cancer Center
University of Rochester
601 Elmwood Ave.
Rochester, NY 14534
USA

VIKTOR BRABEC
Institute of Biophysics
Czechoslovak Academy
of Sciences
Královopolská, 135
612 65 Brno
Czechoslovakia

M. BRAMEZZA
Institute of Clinical Chemistry
Udine General Hospital
33100 - Udine
Italy

G. BRESSA
Dip. di Farmacologia
Università di Padova
Largo Meneghetti, 2
35131 - Padova
Italy

H. BRUNNER
University of Regensburg
D-8400 Regensburg
FRG

D.M. BRYAN
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

A. BURLINA
Cattedra di Chimica e
Microscopia Clinica
Università di Padova
Via Ospedale Civile
35100 - Padova
Italy

JEAN-LUC BUTOUR
CNRS Laboratorie Pharma-Toxicol.
Fondamentales
205, Route de Narbonne
31400 - Toulouse
France

A. CADONI
Istituto di Anatomia Umana
Università di Genova
Genova
Italy

P.P. GAGOL
Istituto di Patologia Chirurgica I
Via N. Giustiniani, 2
Università di Padova
35100 - Padova
Italy

M. CALLIGARIS
Dept. of Chemistry
University of Pavia
27100 - Pavia
Italy

F. CARMIGNOTO
Ospedale Camposampiero
ULSS 20
Via P. Cosma 1
35012 - Camposampiero - Padova
Italy

MARIA CARRARA
Dip. di Farmacologia
Università di Padova
Largo Meneghetti, 2
35131 - Padova
Italy

H. CARRASCO
Dept. of Pathology
The University of Texas
System Cancer Center
M.D. Anderson Hospital and
Tumor Institute
Houston, TX 77030
USA

GIUSEPPE CARTEI
Divisione di Oncologia Medica
Ospedale di Udine
33100 - Udine
Italy

S.G. CARVAJAL
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

E. CATTARUZZI
Institute of Nuclear Medicine
Udine General Hospital
33100 - Udine
Italy

SABINA CAUCI
Dip. di Biochimica, Biofisica e
Chimica Macromolecolare
Università di Trieste
P.le Europa, 1
34127 - Trieste
Italy

HARDY S.O. CHAN
National University of Singapore
Kent Ridge
Singapore, 0511

PETER K.L. CHAN
Dept. of Chemistry
University of British Columbia
Vancouver, B.C., V6T 1T6
Canada

STEPHEN G. CHANEY
Dept. of Biochemistry and Nutrition
School of Medicine
University of North Carolina
Chapel Hill, NC 27514
USA

D.J. CHAPLIN
Cancer Research Centre
601 West 10th Avenue
Vancouver, B.C., V5Z 1L3
Canada

C. CHARNSANGAVEJ
Dept. of Radiology
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
Houston, TX 77030
USA

VALERIA CHERCHI
Dip. di Chimica Inorganica
Metallorganica e Analitica
Università di Padova
Via Loredan, 4
35131 - Padova
Italy

R.G. CHILD
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

ASIT CHOKSI
Dept. of Medical Oncology
The University of Texas
System Cancer Center
M.D. Anderson Hospital and
Tumor Institute
Houston, TX 77030
USA

JEAN-CLAUDE CHOTTARD
Lab. de Chimie et de Biochimie
Pharmacologiques - Toxicologiques -
Paris V
45, rue des Saint-Pères
75270 - Paris
France

LORENZO CIMA
Dip. di Farmacologia
Università di Padova
Largo E. Meneghetti, 2
35131 - Padova
Italy

MICHAEL J. CLARKE
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

ERIK CLAUSEN
Roskilde University
Institute of Life Sciences and Chemistry
P.O. BOX 260
4000 Roskilde
Denmark

N. COLOMBO
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

MARINA COMELLI
Divisione di Oncologia Medica
Ospedale di Udine
33100 - Udine
Italy

ELISA CONTESSI
Divisione di Oncologia Medica
Ospedale di Udine
33100 - Udine
Italy

BENEDETTO CORAIN
Centro di Studio sulla Stabilità e
Reattività dei Composti di
Coordinazione, C.N.R.
Via Marzolo, 1
35131 - Padova
Italy

DANG G. CRACIUNESCU
Dept. of Inorganic and Analytical
Chemistry
Faculty of Pharmacy
Madrid
Spain

D. CROWTHER
Medical Oncology
Christie Hospital
University of Manchester
Oxford Road
Manchester, M13 9PT
UK

SILVIA D'ANCONA
Dip. di Farmacologia
Università di Padova
Largo Meneghetti, 2
35131 - Padova
Italy

P.P. DAPIAN
Istituto di Patologia Chirurgica I
Via N. Giustiniani, 2
Università di Padova
35100 - Padova
Italy

PETER C. DEDON
Dept. of Pharmacology and
Cancer Center
University of Rochester
601 Elmwood Ave
Rochester, NY 14534
USA

B. DE JONG
Dept. of Antropogenetics
University Hospital
Oostersingel, 59
9713 EZ Groningen
The Netherlands

W.H. DE JONG
Lab. Pathology
Nat. Inst. Public Health
3720 BA Bilthoven
The Netherlands

LEO DEN ENGELSE
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

E.G.E. DE VRIES
Dept. of Medical Oncology
University Hospital
Oostersingel, 59
9713 EZ Groningen
The Netherlands

L. DEWIT
The Netherlands Cancer Institute
Dept. Exp. Radiotherapy
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

F.J. DIJT
Dept. of Chemistry
Gorlaeus Lab.
Leiden University
2300 RA Leiden
The Netherlands

I. DIMERY
University of Texas
System Cancer Center
M.D. Anderson Hospital and
Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

FRANCESCO DI RE
Istituto Nazionale per lo Studio e
la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

A. DOADRIO
Dept. of Inorganic and
Analytical Chemistry
Faculty of Pharmacy
Madrid
Spain

L. DOLZANI
Institute of Microbiology
University of Trieste
34127 - Trieste
Italy

SHERYL L. DORAN
Engelhard Corporation
Menlo Park CN-28
Edison, NJ 08818
USA

JAROSLAV DROBNIK
Inst. of Macromolecular Chem. INC
Czechoslovak Acad. Sci.
Heyrovsky Sq. 2
162 06 Prague 6
Czechoslovakia

R. DUBBELMAN
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

NGUYEN-HUY DUNG
Laboratoire de Chimie Minérale
et Structurale
Unité associée au C.N.R.S. n. 200
4, Avenue de l'Observatoire
75270 - Paris
France

F.E. DURR
Lederle Laboratories
Medical Research Division
Pearl River, NY10965
USA

ALAN EASTMAN
Eppley Institute for Research in Cancer
University of Nebraska
Medical Center
42nd and Dewey
Omaha, NE 68105
USA

J. EMONDT
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

K. ENDOH
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

J. ENGEL
Asta - Pharma AG
D-8400 Bielefeld 14
FRG

A. EPIS
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

M. ESPOSITO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

J.M. FADOOL
Dept. of Zoology and Biological Sciences
Michigan State University
East Lansing, MI 48823
USA

O. FAGGIONATO
Istituto di Microbiologia
Università di Padova
Via A. Gabelli, 63
35100 - Padova
Italy

GIUSEPPINA FARAGLIA
Dip. di Chimica Inorganica,
Metallorganica e Analitica
Via Loredan, 4
35131 - Padova
Italy

NICHOLAS P. FARREL
Dept. of Chemistry
University of Vermont
Burlington, VT 05405
USA

GINO FAVARETTO
Ospedale Civile di Padova
Divisione Oncologia Medica
Via Ospedale, 38
35100 - Padova
Italy

T. FELDER
University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holombe Blvd.
Houston, TX 77030
USA

ANNE MARIE J. FICHTINGER-SCHEPMAN
Med. Bio. Lab. TNO
P.O. Box 45, Lange Kleiweg
2280 AA Rijswijh
The Netherlands

MARIO V. FIORENTINO
Ospedale Civile di Padova
Divisione Oncologia Medica
Via Ospedale, 38
35100 - Padova
Italy

G. FLEISHMAN
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
Houston, TX 77030
USA

B. FLOOT
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

PALMARISA FRANCHETTI
Dip. di Scienze Chimiche
Università di Camerino
Via S. Agostino, 1
62032 - Camerino
Italy

H. FRANKLIN
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

GIUSEPPE FRASCHINI
Dept. of Medical Oncology
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

FRANK FRIEDLOS
Dept. Mol. Pharmacology
Inst. of Cancer Research
Sutton, Surrey, SM2 5PX
UK

R.A. FULCO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

A. FURLANI
Istituto di Farmacologia e Farmacognosia
Università di Trieste
34127 - Trieste
Italy

R.D. GALANG
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

P. GALLOTTI
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

A. GAROUFIS
Dept. of Chemistry
University of Ioannina
Ioannina
Greece

O. GEATTI
Institute of Nuclear Medicine
Udine General Hospital
33100 - Udine
Italy

C. GHIOTTO
Ospedale Civile di Padova
Divisione Oncologia Medica
Via Ospedale, 38
35100 - Padova
Italy

GREGORY GIBBONS
Dept. of Biochemistry and Nutrition
School of Medicine
University of North Carolina
Chapel Hill, NC 27514
USA

MARCEL GIELEN
Faculty of Engineering
AOSC Unit
Free University of Brussels
Pleinlaan 2
B-1050 Brussels
Belgium

J.R. GLASS
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

P. GORI
Istituto Medicina del Lavoro
Università di Padova
Via J. Facciolati, 71
35100 - Padova
Italy

ANTHONY GRINGERI
Dept. of Pharmacology
and Cancer Center
University of Rochester
601 Elmwood Ave.
Rochester, NY 14534
USA

RONALD GUST
Lehrstuhl für Pharmazeutische Chemie II
Universität Regensburg
Universitätsstrasse, 31
D-8400 Regensburg
FRG

MILES P. HACKER
Vermont Regional Cancer Center
and Dept. of Pharmacology
University of Vermont
Burlington, VT 05405
USA

NICK HADJILIADIS
Dept. of Chemistry
University of Ioannina
Ioannina
Greece

E. HÁJEK
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

JOHN R. HALL
Dept. of CHemistry
University of Queensland
Brisbane 4067
Australia

T.C. HAMILTON
Medicine Branch
Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20892
USA

M.E. HEIM
Onkologisches Zentrum des Klinikums
Mannheim der Universität Heidelberg
Theodor - Kutzer - Ufer
D-6800 Mannheim 1
FRG

A.P.M. HEINTZ
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

J. HEJL
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

P. HILGARD
Asta - Pharma AG
D-4800 Bielefeld 14
FRG

E. HLADKÁ
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

JOSEPH J. HLAVKA
Lederle Laboratories
Medical Research Division
Middletown Road
Pearl River, NY 10965
USA

B. HOFÍREK
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

EGGEHARD HOLLER
Inst. Biophysik and Physikalische
Biochemie der Universität
Universitässtrasse, 31
D-8400 Regensburg
FRG

STEVEN L. HOLLIS
Engelhard Corporation
Menlo Park CN-28
Edison, NJ 08818
USA

U. HOLZINGER
University of Regensburg
D-8400 Regensburg
FRG

M. HONDA
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

WAUN KI HONG
The University of Texas
System Cancer Center
Dept. of Medical Biology
M.D. Anderson Hospital
and Tumor Institute
Houston, TX 77030
USA

G. HORTOBAGYI
Dept. of Medical Oncology
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
Houston, TX 77030
USA

GEKE A.P. HOSPERS
Dept. of Medical Oncology
University Hospital
Oostersingel, 59
9713 EZ Groningen
The Netherlands

BOB A. HOWELL
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

STEPHEN B. HOWELL
Dept. of Medicine and Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

INTISAR HUSAIN
University of North Carolina
Dept. of Biochemistry
Chapell Hill, NC 27514
USA

B.J. HUTCHINSON
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

KENJI INAGAKI
Dept. of Chemistry
Gorlaeus Lab.
Leiden University
2300 RA Leiden
The Netherlands

L.I. IOZEP
Chemicopharmaceutical Institute
Dept. of Analytical Chemistry
Prof. Popov Street, 14
197022 Leningrad
URSS

B.R. JAMES
Dept. of Chemistry
University of British Colombia
Vancouver, B.C., V6T 1T6
Canada

D.A. JOHNSON
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

NEIL P. JOHNSON
Laboratoire de Pharmacologie et de
Toxicologie Fondamentales du CNRS
205, Route de Narbonne
31077 - Toulouse
France

E. JOOSEN
Faculty of Engineering
AOSC Unit
Free University of Brussels
Pleinlaan 2
B-1050 Brussels
Belgium

J. JOSEPHSEN
Roskilde University
Institute of Life
Sciences and Chemistry
P.O. Box 260
4000 Roskilde
Denmark

K. JURKSCHAT
Faculty of Engineering
AOSC Unit
Free University of Brussels
Pleinlaan 2
B-1050 Brussels
Belgium

GERALD JUST
Institut fur Biophysics Biochem.
Universitatsstr 31
D-8400 Regensburg
FRG

S. KASSELOURI
Dept. of Chemistry
University of Ioannina
Ioannina
Greece

BERNHARD K. KEPPLER
Anorganish-Chemisches Institut
Universität Heidelberg
Im Neuenheimer Feld 270
D-6900 Heidelberg
FRG

G. KERSZMAN
Roskilde University
Institute of Life
Science and Chemistry
P.O. Box 260
4000 Roskilde
Denmark

YOSHINORI KIDANI
Faculty of Pharmaceutical Sciences
Nagoya City University
3-1 Tanabe-dori, Mizuho-Ku
Nagoya 467
Japan

H. KINOSHITA
Chiba Cancer Center
666-2 Nitona-machi, Chiba-shi
Tokyo
Japan

FRANTISEK KISS
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

VLADIMIR KLEINWÄCHTER
Institute of Biophysics
Czecholosvak Academy
of Sciences
Královopolská, 135
612 65 Brno
Czechoslovakia

NORBERT KNEBEL
Institut für Pharmazie
Universität Regensburg
Universitatsstrasse, 31
D-8400 Regensburg
FRG

R.J. KNOX
Dept. Mol. Pharmacology
Inst. of Cancer Research
Sutton, Surrey, 5M2 5PX
UK

MASUO KOIZUMI
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

KRSTO KOLARIC
Central Institute for Tumors
and Allied Diseases
Ilica, 197
4100 - Zagreb
Yugoslavia

M. KOMURA
Shionogi Research Laboratories
Shionogi & Co
Fukushima - Ku
Osaka 553
Japan

PETRA KÖPF-MAIER
Institut für Anatomie
Freie Universität Berlin
Luise Strasse, 15
D-1000 Berlin 33
FRG

IRWIN H. KRAKOFF
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

ALAN KRAMER
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

OLGA KRIZANOVIC
Institut für Anorganische
und Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

R. KUMAR
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

G. LAI
Medicine Branch
Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20892
USA

J.C. LANCELOT
Laboratorie de Chimie Thérapeutique
U.F.R. de Pharmacie
1, rue Vaubénard
14032 - Caen
France

F. LANDONI
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

S.A. LANG, Jr
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

P. LAPETOULE
Laboratoire de Pharmacologie et
de Toxicology Fondamentales du CNRS
205, Route de Narbonne
31077 - Toulouse
France

J.P. LAUSSAC
Laboratoire de Chimie de
Coordination du CNRS
205, Route de Narbonne
31400 - Toulouse
France

EDWIN L.M. LEMPERS
State University Leiden
Gorlaeus Lab.
P.O. Box 9502
2300 RA Leiden
The Netherlands

MARC LENG
Centre Biophysique Moleculaire
CNRS
Avenue Recherche Scientifique
45071 - Orleans
France

E. LEPRI
Istituto di Farmacologia
Università di Perugia
Via del Giochetto
06100 - Perugia
Italy

Y-I LIN
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

M.J. LIND
Medical Oncology
Christie Hospital
University of Manchester
Oxford Road
Manchester, M13 9 PT
UK

H.L. LINDSAY
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

BERNHARD LIPPERT
Institut für Anorganische
und Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

CHARLES L. LITTERST
National Cancer Institute
National Institute of Health
Bethesda, MD 20892
USA

BRUNO LONGATO
Centro di Studio sulla Stabilità e
Reattività dei Composti di
Coordinazione, C.N.R.
Via Marzolo, 1
35131 - Padova
Italy

JAMES R. LUSTY
School of Chemistry
Lancashire Polytechnic
Preston, PR1 2TQ
UK

FRANZ LUX
Institut Radiochemie der Technischen
Universität Munchen
Walther Meisner Str. 3
D-8046 Garching
FRG

D.A. LYDALL
Dept. Mol. Pharmacology
Inst. of Cancer Research
Sutton, Surrey, SM2 5PX
UK

GIANNA MAGNOLFI
Dip. di Farmacologia
Università di Padova
Largo Meneghetti, 2
35131 - Padova
Italy

HISASHI MAJIMA
Chiba Cancer Center
666-2 Nitona-machi, Chiba-shi
Chiba Tokyo
Japan

I. YU. MAMELKINA
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

T. MANCILLA
Faculty of Engineering
AOSC Unit
Free University of Brussels
Pleinlaan 2
B-1050 Brussels
Belgium

COSTANTINO MANGIONI
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

STEPHEN C. MANN
Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

LUIGI G. MARZILLI
Emory University
Dept. of Chemistry
Atlanta, GA 30322
USA

H. MASUDA
Medicine Branch
Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20892
USA

T. MATSUMOTO
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima - Ku, Tokyo 171
Japan

STANLEY K. MAULDIN
Dept. of Biochemistry and Nutrition
School of Medicine
University of North Carolina
Chapel Hill, NC 27514
USA

CHARLES A. Mc AULIFFE
Dept. of Chemistry
U.M.I.S.T.
Manchester, M60 1QD
UK

K. Mc CARTHY
University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

J.G. Mc VIE
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

M. MERLANO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

GIOVANNI MESTRONI
Dip. di Scienze Chimiche
Università di Trieste
P.le Europa, 1
34127 - Trieste
Italy

WOLFGANG MICKLITZ
Institut für Anorganische
und Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

H. MITSUI
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

MADAN MOHAN
Dept. of Chemistry
N.R.E.C. College
Khurja 203131 (U.P.)
India

E. MONTI
Istituto di Farmacologia
Via Celoria
20133 - Milano
Italy

CARLO MONTI-BRAGADIN
Istituto di Microbiologia
Università di Trieste
Via Fleming, 22
34127 - Trieste
Italy

THOMAS J. MONTINE
Dept. of Pharmacology
and Cancer Center
University of Rochester
601 Elmwood Ave.
Rochester, NY 14534
USA

FRANCA MORAZZONI
Dip. Chimica Inorganica e
Metallorganica
Via Venezian, 21
20133 - Milano
Italy

K. MORIKAWA
New Drug Research Laboratories
Chugai Pharmaceutical Co.
Takada 3-41-8
Toshima-Ku, Tokyo 171
Japan

N.H. MULDER
Dept. of Medical Oncology
University Hospital
Oostersingel, 59
9713 EZ Groningen
The Netherlands

G. NARDIN
Dip. di Scienze Chimiche
Università di Trieste
P.le Europa, 1
34127 - Trieste
Italy

E. NASINI
Dip. di Scienze Chimiche
Università di Camerino
Via S. Agostino, 1
62032 - Camerino
Italy

R. NEWMAN
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

MARINO NICOLINI
Dip. di Scienze Farmaceutiche
Università di Padova
Via Marzolo, 5
35131 - Padova
Italy

GIUSEPPE NOCENTINI
Istituto di Farmacologia
Università di Perugia
Via del Giochetto
06100 - Perugia
Italy

J. NOVOTNÝ
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

S. ORIANA
Istituto Nazionale per lo Studio
e la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

ROBERT F. OZOLS
National Cancer Institute
National Institute of Health
Build 10
Bethesda, MD 20892
USA

ADRIANO PACCAGNELLA
Divisione Oncologica
Ospedale Civile
Via Ospedale, 38
35100 - Padova
Italy

JIMMY D. PAGE
University of North Carolina
Dept. of Biochemistry
Chapel Hill, NC 27514
USA

TAMMY PAGE KLINE
Dept. of Chemistry
Emory University
Atlanta, GA 30322
USA

C.D. PALEARI
Cattedra di Chimica e
Microscopia Clinica
Università di Padova
Via Ospedale Civile
35100 - Padova
Italy

A. PAPAIOANNOU
Istituto di Farmacologia e Farmacognosia
Università di Trieste
Via Valerio, 32
34127 - Trieste
Italy

I.S. PAPPAS
Lab. of Pharmacology
Dept. of Pharmaceutical Sciences
University of Thessaloniki
54006 - Thessaloniki
Greece

L. PARACCHINI
Istituto di Farmacologia
Via Celoria
20133 - Milano
Italy

J. PAREDES
The University of Texas
System Cancer Center
M.D. Anderson Hospital
and Tumor Institute
1515 Holcombe Blvd.
Houston, TX 77030
USA

HENRIK B. PEDERSEN
Roskilde University
Institute of Life Sciences and Chemistry
P.O. Box 260
4000 Roskilde
Denmark

S. PELL
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

L. PELLICCIARI-BOLLINI
Dip. di Chimica Inorganica e
Metallorganica
Via Venezian, 21
20133 - Milano
Italy

M.F. PERA
Radiotheraphy Research Unit
Inst. of Cancer Research
Sutton, Surrey, SM 2 5PX
UK

M. PERACCHI
Istituto di Microbiologia
Università di Padova
Via A. Gabelli, 63
35100 - Padova
Italy

F. PICCININI
Istituto di Farmacologia
Via Celoria
20133 - Milano
Italy

DONALD H. PICKER
Pharmaceutical Research
Johnson Matthey Inc.
1401 King Road
West Chester, PA 19380
USA

P.L. PILATI
Istituto di Patologia Chirurgica I
Via N. Giustiniani, 2
Università di Padova
35100 - Padova
Italy

GIUSEPPE PILLONI
Dip. di Chimica Inorganica
Metallorganica e Analitica
Università di Padova
Via Loredan, 4
35131 - Padova
Italy

J. POHL
Asta-Pharma AG
D-4800 Bielefeld 14
FRG

M.C. POIRIER
Laboratory of Cellular
Carcinogenesis and Tumor Promotion
Div. of Cancer Etiology
National Cancer Institute
Bethesda, MD 20892
USA

P. PRONZATO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

F. QUADRIFOGLIO
Institute of Biology
University of Udine
33100 - Udine
Italy

A. RAHMOUNI
Centre de Biophysique Moléculaire
du CNRS
1A, Avenue de la Recherche
45071 - Orléans
France

STEPHEN F. RALPH
Dept. of Chemistry
University of Queensland
Brisbane 4067
Australia

R. RASHIDIANFAR
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

L. RAVALICO
Istituto di Farmacologia e Farmacognosia
Università di Trieste
34127 - Trieste
Italy

HONORAT RAZAKA
Laboratoire de Pharmacologie et de
Toxicologie Fondamentales du CNRS
205, Route de Narbonne
31077 - Toulouse
France

L. REDAELLI
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

EDDIE REED
National Cancer Institute
Bldg 31
National Institute of Health
Bethesda, MD 20892
USA

JAN REEDIJK
Deept. of Chem.
Gorlaeus Lab.
Leiden University
2300 RA Leiden
The Netherlands

G. REZNIK
Institut für Pathologie und Toxikologie
der Fa. Byk Gulden
Friedrich-Ebert-Dann 101
D-2000 Hamburg 70
FRG

M. ROBBA
Laboratoire de Chimie Thérapeutique
U.F.R. de Pharmacie
1, rue Vaubénard
14032 - Caen
France

JOHN D. ROBERTS
Dept. of Medicine
University of Vermont
Room E318, Given Medical Building
Burlington, VT 05405
USA

JOHN J. ROBERTS
Institute of Cancer Research
Clifton Avenue
Sutton, Surrey, SM2 5PX
UK

V.M. RODRIGUEZ
Dept. of Chemistry
Boston College
Chestnut Hill, MA 02167
USA

C. ROOBOL
Faculty of Medicine and Pharmacy
Free University of Brussels
Laarbeeklaan 103/E
B-1090 Brussels
Belgium

RICCARDO ROSSO
Istituto Nazionale per la
Ricerca sul Cancro
Viale Benedetto XV
16132 - Genova
Italy

PETER J. SADLER
Dept. of Chemistry
Birkbeck College
University of London
Malet Street
London, WC1E 7HX
UK

AZIZ SANCAR
School of Medicine Dept. Biochem.
University of North Carolina
Chapel Hill, NC 27514
USA

R. SANGA
Dept. of Medicine and Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

K. SATO
Shionogi Research Laboratories
Shionogi & Co.
Fukushima - Ku
Osaka 553
Japan

GIANNI SAVA
istituto di Farmacologia e Farmacognosia
Università di Trieste
Via A. Valerio, 32
34127 - Trieste
Italy

MASUMI SAWADA
Dept. of Ginecology
The Center for Adult Diseases
1-3-3 Nakamichi Higashinari
Osaka 537
Japan

VITO SCARCIA
Istituto di Farmacologia e Farmacognosia
Università di Trieste
Via Valerio, 32
34127 - Trieste
Italy

W. SCHALLER
Inst. Biophysik und physikalische
Biochemie der Universitat
Universitasstrasse, 31
D-8400 Regensburg
FRG

D. SCHMÄHL
Institut für Toxicologie und
Chemotherapie am Deutschen
Krebsforschungszentrum
Im Neuenheimer Feld 280
D-6900 Heidelberg
FRG

M. SCHMIDT
University of Regensburg
D-4800 Regensburg
FRG

HELMUT SCHOLLHORN
Sektion fur Röntgen und
Elektronenbeugung
Universität Ulm
D-7900 Ulm
FRG

HELMUT SCHÖNENBERGER
Lehrsuhl für Pharmazeutische Chemie II
Universität Regensburg
Universitatsstrasse, 31
D-8400 Regensburg
FRG

N. SCHULTE
Eppley Institute for Research in Cancer
University of Nebraska
Medical Center
42nd and Dewey
Omaha, NE 68105
USA

W. SCHUMACHER
Asta - Pharma AG
D-4800 Bielefeld 14
FRG

A. SCHWARTZ
Centre de Biophysique
Moléculaire du CNRS
1A, Avenue de la Recherche
45071 - Orléans
France

FRANK SCHWARZ
Institut für Anorganische und
Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

VANESSA G. SCHWEITZER
Dept. of Otolaryngology HNS
Henry Ford Hospital
2799 West Grand Blvd.
Detroit, MI 48202
USA

L.B. SELDERKHANOVA
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

S.K. SHAKHATREH
Dept. of General and
Inorganic Chemistry
University of Thessaloniki
54006 - Thessaloniki
Greece

H.L. SHARMA
University of Manchester
Dept. of Medical Biophysics
Oxford Road
Manchester, M13 9PT
UK

N. SHEIBANI
Eppley Institute for Research in Cancer
University of Nebraska
Medical Center
42nd and Dewey
Omaha, NE 68105
USA

O. SHIRATORI
Shionogi Research Laboratories
Shionogi & Co
Fukushima - Ku
Osaka 553
Japan

LIVIA SINDELLARI
Dip. di Chimica Inorganica,
Metallorganica e Analitica
Università di Padova
Via Loredan, 4
35131 - Padova
Italy

SERGIO SITRAN
Istituto di Chimica e Tecnologia dei
Radioelementi
CNR - ICTR
C.so Stati Uniti, 4
35100 - Padova
Italy

KIRSTEN A. SKOV
B.C. Cancer Research Centre
601 West 10th Avenue
Vancouver, B.C., V5Z 1L3
Canada

D.B. SMITH
Medical Oncology
Christie Hospital
University of Manchester
Oxford Road
Manchester, M13 9 PT
UK

ALBERTO SOBRERO
Istituto Nazionale per la Ricerca
sul Cancro
Viale Benedetto IV
16132 - Genova
Italy

C.M. SORENSON
Eppley Institute for Research in Cancer
University of Nebraska
Medical Center
42nd and Dewey
Omaha, NE 68105
USA

G.B. SPATTI
Istituto Nazionale per lo Studio
e la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

CHRISTINE SPELLMEYER FOUTS
Dept. of Chemistry
Emory University
Atlanta, GA 30322
USA

THILO SPRUSS
Institut für Pharmazeutische Chemie II
Universität Regensburg
Universitatsstrasse, 31
D-8400 Regensburg
FRG

S. STEFANELLI
Istituto di Microbiologia
Università di Padova
Via A. Gabelli, 63
35100 - Padova
Italy

ERIC W. STERN
Engelhard Corporation
Menlo Park CN-28
Edison, NJ 08818
USA

ANASTASIYA I. STETSENKO
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
USSR

F.A. STEWART
The Netherlands Cancer Institute
Dept. Exp. Radiotherapy
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

R. SUPINO
Istituto Nazionale per lo Studio
e la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

M. TAMARO
Institute of Microbiology
University of Trieste
34127 - Trieste
Italy

MICHELE TEDESCHI
Boehringer Biochemia Robin
Via Uguzzone, 5
20126 - Milano
Italy

W.W. TEN BOKKEL HUININK
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

PHILIPPE TERHEGGEN
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

U. THEWALT
Sektion Röntgen und
Elektronenbeugung
Universität Ulm
D-7900 Ulm
FRG

J.P.THOMAS
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

L.S. TIKHONOVA
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

NIGEL D. TINKER
U.M.I.S.T.
Dept. of Chemistry
Manchester, M60 IQD
UK

SERGIO TOGNELLA
Boehringer Biochemia Robin
Via S. Uguzzone, 5
20126 - Milano
Italy

W. TORRI
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

C. TOSO
Institute of Pathologic Anatomy
Udine General Hospital
33100 - Udine
Italy

TETSUSHI TOTANI
Shionogi Research Laboratories
Shionogi & Co
Fukushima-Ku
Osaka 553
Japan

SUSANNE TREBERT HAEBERLIN
Institut für Radiochemie der Technischen
Universität Munchen
Walther Meisner Str. 3
D-8046 Garching
FRG

LUCIO TRINCIA
Dip. di Chimica Inorganica,
Metallorganica e Analitica
Università di Padova
Via Loredan, 4
35131 - Padova
Italy

GABRIELE TRÖTSCHER
Institut für Anorganische und
Analytische Chemie
Universität Freiburg
D-7800 Freiburg
FRG

ANNETTE TROY
Andrulis Research Corporation
11800 Baltimore Ave.
Beltsville, MD 20705
USA

A.S. TSIFTSOGLOU
Lab. of Pharmacology
Dept. of Pharmaceutical Sciences
University of Thessaloniki
54006 - Thessaloniki
Greece

CONSTANTINOS A. TSIPIS
Dept. of General and Inorganic
Chemistry
University of Thessaloniki
54006 - Thessaloniki
Greece

A.A. TULUB
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

P. TVARŮŽEK
Research Inst. Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

GIOVANNI VALLE
Centro di Studi sui Biopolimeri, C.N.R.
Via Marzolo, 1
35131 - Padova
Italy

A.T. VAN OOSTEROM
Dept. of Oncology
Antwerpen Univ. Hospital
2520 Edegem
Belgium

L. VASSENA
V Clinica Ostetrico-Ginecologica
Università di Milano
Ospedale S. Gerardo
Via Solferino, 16
Monza, Milano
Italy

SRIHARSHA VELURY
Dept. of Medicine and Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

J. VICK
Dept. of Medicine and Cancer Center
University of California
San Diego, La Jolla, CA 92093
USA

W.V.D. VIJGH
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

B. VIOSSAT
Laboratoire de Chemie Générale
U.F.R. de Médecine et Pharmacie
34, rue du Jardin des Plantes
86034 - Poitiers
France

S. VITTORI
Dip. di Scienze Chimiche
Università di Camerino
Via S. Agostino, 1
62032 - Camerino
Italy

R. VOEGELI
Asta - Pharma AG
D-4800 Bielefeld 14
FRG

G. VOLTAREL
Dip. di Chimica Inorganica,
Metallorganica e Analitica
Università di Padova
Via Loredan, 4
35131 - Padova
Italy

ERWIN VON ANGERER
Universität Regensburg
Institut für Pharmazie
Universitatsstrasse, 31
D-8400 Regensburg
FRG

J. VORLÍČEK
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

OLDŘICH VRANA
Institute of Biophysics
Czechoslovak Academy
of Sciences
Královopolská, 135
612 65 Brno
Czechoslovakia

R.E. WALLACE
Lederle Laboratories
Medical Research Division
Pearl River, NY 10965
USA

E.W. WALLES
Dept. of Chemistry
Central Michigan University
Mt. Pleasant, MI 48859
USA

R. WILLEM
Faculty of Engineering
AOSC Unit
Free University of Brussels
Pleinlaan 2
B-1050 Brussels
Belgium

DAVID R. WILLIAMS
Dept. of Applied Chemistry
University of Wales
P.O. Box 13
Cardiff, CF1 3XF
UK

K.I. YAKOVLEV
Dept. of Analytical Chemistry
Chemicopharmaceutical Institute
Prof. Popov Street, 14
197022 Leningrad
URSS

ROBERT C. YOUNG
Medicine Branch Division
of Cancer Treatment
National Cancer Institute
Bethesda, MD 20892
USA

S. ZAMPIRON
Dip. di Farmacologia
Università di Padova
Largo E. Meneghetti, 2
35131 - Padova
Italy

M. ZANINOTTO
Cattedra di Chimica e
Microscopia Clinica
Università di Padova
Via Ospedale Civile
35100 - Padova
Italy

I. ZÁVODNÁ
Research Institute of Pure Chemicals
Lachema n.e.
621 33 Brno
Czechoslovakia

A. ZICCA
Istituto di Anatomia Umana
Università di Genova
Genova
Italy

J.G. ZIJLSTRA
Dept. of Medical Oncology
University Hospital
Oostersingel, 59
9713 EZ Groningen
The Netherlands

GERALD ZON
Mol. Pharmacology Lab.
Food and Drug Administration
Bethesda, MD 20892
USA

SONIA ZORZET
istituto di Farmacologia e Farmacognosia
Università di Trieste
Via A. Valerio, 32
34127 - Trieste
Italy

FRANCO ZUNINO
Istituto Nazionale per lo Studio
e la Cura dei Tumori
Via Venezian, 1
20133 - Milano
Italy

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To my wife PAOLA
and my sons CLAUDIO and BENEDETTA.

A: Biochemistry of Platinum Complexes in Cancer Chemotherapy.

KINETICS OF FORMATION OF GENOTOXIC PLATINUM-DNA LESIONS *IN VIVO*

N.P. Johnson, P. Lapetoule, H. Razaka and J.L. Butour

INTRODUCTION

Pascoe and Roberts (1) first observed DNA-DNA interstrand crosslinks in cultured HeLa cells which had been treated with cisplatin using density gradient techniques. Zwelling and coworkers (2) subsequently showed using alkaline elution that interstrand crosslinks formed by cis-DDP (cis-$PtCl_2(NH_3)_2$) in L1210 leukemia cells evolved as a function of time during post-treatment incubation and reached a maximum 6-12 h after treatment. These results have been confirmed in cultured human fibroblasts treated with cisplatin by means of alkaline elution and DNA renaturation techniques (3). The appearance of DNA interstrand crosslinks during post-treatment incubation is thought to be the result of the transformation of monofunctional platinum-DNA adducts into bifunctional lesions (4).

However this interpretation has been critisized by Pinto and Lippard (5) who pointed out that intracellular cisplatin may continue to bind to DNA during post-treatment incubation thereby resulting in increased crosslink formation. Alternatively cis-DDP itself may not be responsible for the inhibitory activity. Rather slowly formed metabolites of the drug might react with DNA and block its replication (6,7). In either case DNA binding would increase during post-treatment incubation.

In order to test this hypothesis we measured DNA binding during the first few hours after treatment of exponentially growing L1210 cells which had been treated with 20 μM cisplatin. The observed DNA binding immediately after treatment was 2×10^{-5} platinum per

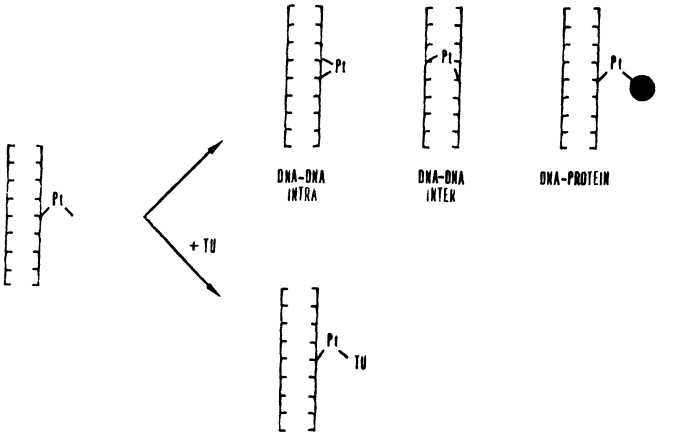

Fig. 1 Monoadduct quenching. Monofunctional platinum-DNA adducts may form intrastrand and interstrand DNA crosslinks and protein-DNA crosslinks in the cell. Thiourea reacts with monofunctional adducts and prevents the formation of these crosslinks.

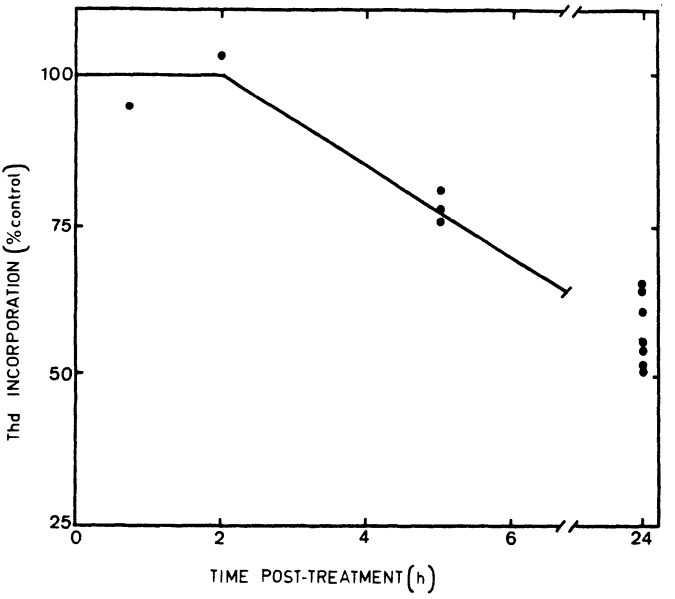

Fig. 2 Relative [^{3}H]-Thd incorporation into L1210 leukemia cells following 1h treatment with 18 μM cisplatin. Experimental conditions as in ref. 28.

nucleotide. This level of binding decreased during 5 h post-treatment incubation to $1x10^{-5}$ platinum per nucleotide. This decrease occured in the absence of cell proliferation and may reflect a DNA repair process. Similarly, measurement of DNA binding in exponentially growing V79 cells treated with cisplatin showed 34 % loss of platinum from the cellular DNA after 10 h post-treatment incubation (8). To our knowledge increased binding of platinum to DNA during post-treatment incubation of mammalian cells treated with cisplatin has never been reported. Although an equilibrium between the formation and repair of platinum-DNA lesions can not be excluded by these results, the concentration of platinum on cellular DNA nevertheless appears to be at its maximum value immediately after treatment with cisplatin.

Monoadduct quenching has proved to be a powerful tool to investigate the evolution of platinum-DNA adducts during post-treatment incubation (9). This method employs a sulphur donor ligand, thiourea, which irreversibly binds to the monofunctional platinum-DNA adducts thereby blocking the formation of bifunctional lesions. Exposure of mammalian cells to thiourea immediately after treatment with low doses of cis-DDP decreases the mutagenic and toxic effects of this drug (4,9,10). Delayed application of thiourea permits the formation of crosslinks and leads to increased toxicity and mutagenicity ; after several hours, thiourea has no influence on the genotoxic effects of cisplatin.

It is unlikely that thiourea inhibits crosslink formation by preventing unmodified intracellular cisplatin from reacting with DNA during post-treatment incubation. Such a mechanism would require that in the absence of thiourea the level of platinum on the cellular DNA should increase after removal of exogenous drug which is not observed. In addition, nontoxic doses of thiourea do not reverse the formation of platinum-DNA crosslinks in cells (9) which indicates that thiourea does not remove platinum from cellular DNA under these conditions. The best explanation of available data is that thiourea blocks the formation of bifunctional adducts (such as interstrand crosslinks) during post-treatment incubation by reaction with the

second arm of the monofunctional adduct (Fig. 1, after ref. 4).

If this analysis is correct, monoadduct quenching may be used to investigate the influence of the slow-forming bifunctional lesions (those which appear during the first few hours after treatment with cisplatin) on survival, growth inhibition and mutagenesis (which are measured several cell cycles after treatment). The time after treatment in which toxicity and mutagenicity are sensitive to thiourea give a rough idea of the kinetics of formation of the biologically important DNA lesions. Knowledge of the kinetics of formation of these lesions may be useful to distinguish the molecular events responsible for the different genotoxic effects of cisplatin. For example, thiourea loses its effect on the mutagenicity of cisplatin in V79 cells after 4.5 hours post-treatment incubation. Under these conditions, sister chromatid exchange can still be antagonised by thiourea which led Bradley and coworkers (10) to suggest that different lesions may be responsible for these two phenomena. We have used monoadduct quenching to measure the kinetics of formation of lesions responsible for several biochemical and pharmacological effects of cisplatin.

INHIBITION OF DNA SYNTHESIS IN L1210 CELLS

DNA replication (as determined by Thd incorporation) can be measured immediately after treatment with platinum compounds and may be sensitive to time-dependent modifications of the DNA template. Thd incorporation in mammalian cells treated with low concentrations of cis-DDP decreases during the first few hours post-treatment (6,7). For example, Thd incorporation does not diminish immediately after treatment of L1210 cells for 1 h with 18 μM cis-DDP but subsequently decreases during incubation in the absence of drug (Fig. 2).

Cells treated with low concentrations of cis-DDP may stop dividing after completing the current round of replication and the progressive loss of Thd incorporation might be the result of increasing numbers of cells encountering a G2 block (11-13). In this case, decreased Thd incorporation in mammalian cells treated with cisplatin may not necessarily be a consequence of DNA damage but might reflect

other lesions which could delay the entrance of cells into S phase. Kanno and coworkers (14) have examined this hypothesis in unsynchronized mouse mammary carcinoma cells by measuring Thd incorporation and the onset of G2 block by means of flow cytometry. Cells treated for 1 h with 20 μM cisplatin pass normally through the first cell cycle and G2 arrest occurs 18 h after treatment. In contrast Thd incorporation is reduced dramatically in the first 6 h after exposure to cisplatin. The different kinetics for the onset of these two phenomena suggest that, at least in this cell line, decreased Thd incorporation developes independently of cell cycle block.

Alternatively the slow formation of interstrand DNA crosslinks may be responsible for the onset of inhibition of DNA synthesis during post-treatment incubation (15). In order to investigate this hypothesis, we have looked at the effect of monoadduct quenching on the inhibition of Thd incorporation in L1210 cells treated with cis-DDP. When a 1 h pulse of 50 mM thiourea was applied 45 min after treatment with cisplatin (the time necessary to wash the cells and place them in drug-free media) the effect of the drug was entirely reversed (Fig. 3). Between 3-6 h after treatment, the effect of thiourea decreased and 6 h after treatment thiourea no longer antagonized the inhibition of Thd incorporation by cisplatin.

It is worth noting that when cells were treated with 18 μM cis-DDP, the entire onset of inhibition of DNA synthesis occured during post-treatment incubation in the absence of drug (Fig. 2). Thiourea completely reversed the inhibition of DNA synthesis in cells treated at this dose (Fig. 3). Hence, it appears that the lesions formed during post-treatment incubation are both necessary and sufficient for the inhibition of DNA synthesis.

GROWTH INHIBITION

Growth inhibition (measured by cell proliferation 48 hours after treatment with cis-DDP) could be partially reversed by incubation of the cells for 1 hour with media containing 50 mM thiourea if the thiourea was applied immediately after treatment by the platinum compound. For example, cells treated for 1 h with 18 μM cis-DDP exhibited only 20 % as many cells as the control culture whereas

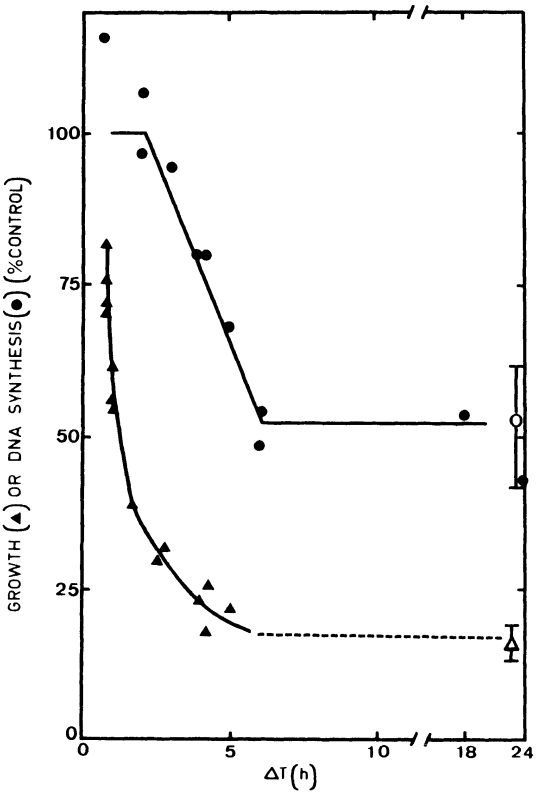

Fig. 3 Effect of thiourea on Thd incorporation and cell proliferation. L1210 leukemia cells were treated as in Fig. 2 and a 1h pulse of 50 mM thiourea was applied at various times ΔT after removal of cisplatin. Thd incorporation was measured 24h after cisplatin treatment and cell numbers determined after 48h. Open symbols are the inhibitory effect of cisplatin without addition of thiourea.

exposure of cells to thiourea 45 min after treatment increased cell growth to 75 % of control values (Fig. 3). As the pulse of thiourea was delayed, the compound lost its capacity to restore cell proliferation. The similar time-dependent effect of thiourea on cell proliferation and inhibition of DNA synthesis suggests that these two phenomena may be the result of the formation of identical platinum-DNA lesions during post-treatment incubation.

DELAYED MUTAGENESIS IN BACTERIA

We have recently discovered that the mutation frequency of bacteria treated with cis-DDP may increase during post-treatment incubation in non-permissive conditions. Exponentially growing E. coli were treated with 60 μM cis-DDP for 2 h, washed and incubated at 37°C in 10 mM $MgSO_4$. Colony forming ability (30 ± 5 % of control cells) did not change during post-treatment incubation. The mutation frequency (his reversion to prototrophy) was 35 ± $10x10^{-8}$ immediately after treatment with 60 μM cis-DDP as previously reported (16). The number of revertants doubled during the first hour post-treatment incubation and reached a plateau of 115 ± $10x10^{-8}$ after 3 h (Fig. 4).

This increased mutation frequency was not due to fixation of additional platinum on the bacterial DNA during post-treatment incubation. Bacteria were washed after 2 h treatment until no platinum was observed in the supernatant by flameless atomic absorption. DNA was isolated by phenol extraction (16) and the molar ratio of platinum per nucleotide immediately after treatment ($2.6 \pm 0.3x10^{-4}$) was identical after 4 h post-treatment incubation ($2.4 \pm 0.2x10^{-4}$).

Monoadduct quenching blocks the post-treatment augmentation of mutagenesis in E. coli treated with cis-DDP (Fig. 5). Fifty mM thiourea did not alter the survival or mutation frequency of untreated bacteria and did not modify the survival of cultures which had been treated with 60 μM cis-DDP. However, exposure of bacteria to 50 mM thiourea immediately after treatment with cisplatin completely inhibited the observed increase in mutagenicity. Application of thiourea after 2 h post-treatment incubation inhibited further increase in the number of mutants whereas, after 3 h, thiourea had no effect. Hence thiourea blocks but does not reverse the post-treatment

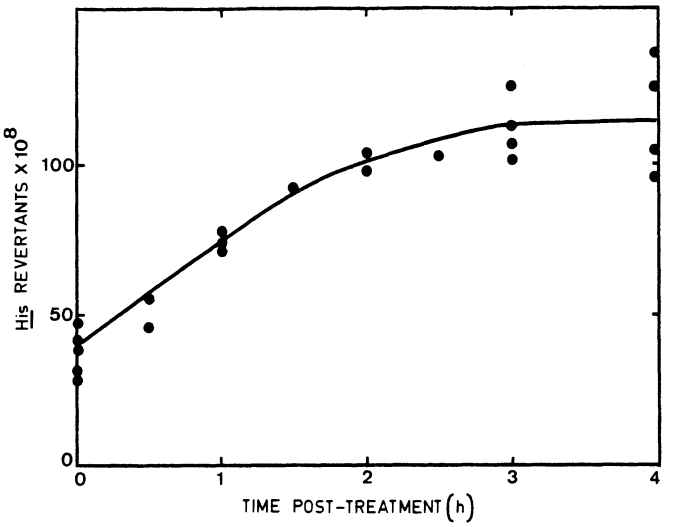

Fig. 4 Increased mutation frequency during post-treatment incubation of E.coli in non-permissive conditions. Bacteria were treated with 60 µM cis-DDP for 2h, washed and resuspended in 10 mM $MgSO_4$ at 37°C. Aliquots were taken at various times and mutation frequency determined as in ref. 16.

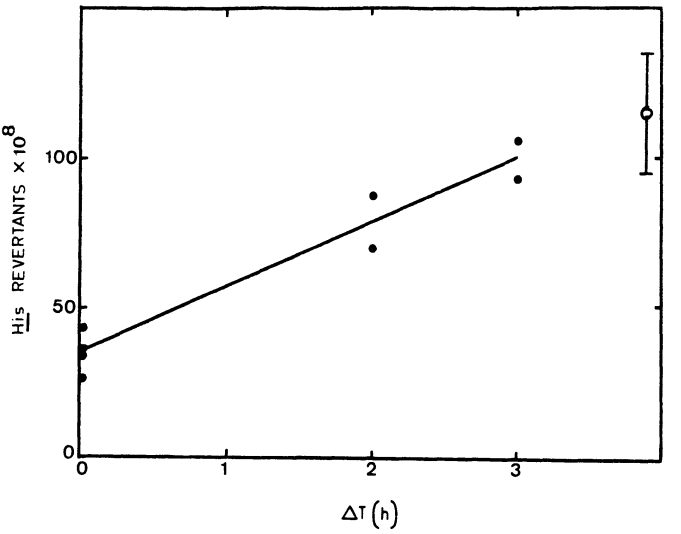

Fig. 5 Effect of monoadduct quenching on post-treatment mutagenesis in E.coli . Bacteria which had been treated as in Fig. 4 were exposed to 50 mM thiourea at various times ΔT after removal of cisplatin. Cells were plated for measurement of mutation frequency after 4h post-treatment incubation. Open symbol ○ mutagenesis in the absence of thiourea.

mutagenesis of cisplatin in E. coli.

Reversion of bacteria to prototrophy by platinum compounds requires the SOS response, an inducible repair process under the control of the lexA and recA genes which permits the bacterial replication machinery to bypass certain DNA damage (17 and refs. therein). RecA protein is maximally induced after 2 h exposure of bacteria to cis-DDP and remains elevated for several hours in drug-free media (18). RecA protein is induced by DNA lesions which block the replication fork (19) and mutations are known to occur as the result of certain bifunctional platinum-DNA intrastrand crosslinks (20,21) which may inhibit DNA replication (22). In contrast, monofunctional platinum-DNA adducts such as those formed by [PtCl(dien)]Cl do not block replication and are not toxic or mutagenic in bacteria (23 ; unpublished). These considerations suggest a possible explanation for the increased mutation frequency that we observe during post-treatment incubation. Immediately after treatment a certain population of bacteria with monofunctional platinum-DNA lesions may be able to replicate normally to produce colonies with the his phenotype. During 4 h incubation, these monofunctional lesions may evolve into bifunctional DNA adducts which undergo SOS repair, thereby leading to mutations and the reversion to prototrophy.

ANTITUMOR ACTIVITY AGAINST L1210 LEUKEMIA

Finally we wanted to know whether or not the lesions responsible for the antitumor activity of cisplatin exhibit a similar time-dependent sensitivity to monoadduct quenching as cytotoxicity and the inhibition of replication (Fig. 3). Burchenal and coworkers (24) have reported that pretreatment of mice with thiourea inhibits the antitumor effect of cisplatin toward L1210 leukemia. Alternatively, cultured L1210 leukemia cells may be treated ex vivo prior to implantation in the mouse. This approach has been previously exploited to show that the antitumor activity of an analogue of cisplatin, cis-bis(cyclopentylamine)dichloroplatinum(II), toward murine leukemia is primarily the result of cytotoxic effects of the drug and does not require the host immune response (25). Treatment ex vivo of L1210 cells for 1 h with 30 μM cis-DDP prior to i.p. implantation of the

cells in mice produces an increased life span of 53 % (Table 1). As a curiosity, we note in passing that treatment of L1210 leukemia cells ex vivo with 100 μM trans-DDP produces a similar increased life span which suggests that the lack of antitumor activity for the trans compound may be due in part to its toxicity toward the animal. Exposure of cells to 50 mM thiourea immediately after treatment with cisplatin (ΔT=0) completely reverses the antitumor activity in these experiments. If the pulse is delayed for 5 h, (ΔT=5) monoadduct quenching has no effect. These results suggest that lesions which are formed during post-treatment incubation are likely responsible for the antitumor activity of cisplatin toward L1210 leukemia in mice.

Table 1. Antitumor activity, T/C (%), against L1210 leukemia cells treated ex vivo with 30 μM cisplatin.

no thiourea	50 mM thiourea	
	Δ T=0	Δ T=5 h
153 ± 10	111 ± 2	138 ± 6

Between 0.5-1.0x10^5 L1210 leukemia cells were incubated 1 h with 30 μM cis-DDP in serum-free RPMI media. In some experiments cells were exposed to 50 mM thiourea in RPMI media either immediately after treatment (Δ T=0) or 5 h later (Δ T=5). Cells were subsequently implanted i.p. in mice and the life span of animals with treated cells (T) is reported as a percentage of the life span of animals with untreated cells (C) which died 10 days after implantation. Uncertainty is the range of 2-3 independent experiments.

It is clear from numerous experiments with prokaryotes and eukaryotes that cisplatin binds to cellular DNA and blocks replication (for a review see 26). However, the proof that this mechanism is responsible for the antitumor activity rests largely on indirect arguments (such as the correlation between antitumor activity and genotoxic effects for a series of Pt(II) complexes) or arguments by analogy with other bifunctional drugs such as alkylating agents. Monoadduct quenching allows the measurement of the kinetics of formation of lesions which are responsible for the inhibition of replication and for the antitumor effect of cisplatin in L1210 cells (Fig. 3, Table 1). Both phenomena arise from lesions which lose

their sensitivity to thiourea during post-treatment incubation. These results suggest that slow-forming platinum-DNA lesions may be responsible for the cytotoxic and antitumor activity of cisplatin against L1210 leukemia.

CONCLUSION

In summary, post-treatment exposure to thiourea restores DNA synthesis and proliferation of L1210 cells which have been treated with cisplatin (Fig. 3). Monoadduct quenching also restores colony forming ability in these cells (9) and inhibits the antitumor activity of this drug after treatment of cells ex vivo (Table 1). In all cases thiourea antagonises the biological effects of cisplatin if cells are exposed immediately after treatment with the platinum compound and it loses its effect if thiourea rescue is delayed.

Measurement of the quantity of platinum on cellular DNA indicates that DNA binding does not increase during post-treatment incubation. Hence thiourea probably does not exert its effect by acting as a sponge which soaks up intracellular cisplatin or a metabolite prior to its reaction with DNA. Thiourea reacts in vitro with monofunctional platinum-DNA adducts thereby inhibiting the formation of bifunctional lesions (27). Thiourea also blocks the formation of interstrand DNA crosslinks in mammalian cells by cisplatin (9). Hence the reaction of thiourea with the second arm of the monofunctional platinum-DNA adduct (4) is likely responsible antagonism of the biological effects of cisplatin described in this paper.

The time-dependent disappearance of thiourea rescue during post-treatment incubation can be used to measure the kinetics of formation of genotoxic platinum-DNA lesions in the cell. The kinetics of formation of these lesions and their susceptibility to thiourea might be useful criteria for distinguishing pharmacologically important DNA damage.

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THE ROLE OF PLATINUM-DNA INTERACTIONS IN THE CELLULAR TOXICITY AND ANTI-TUMOUR EFFECTS OF PLATINUM CO-ORDINATION COMPOUNDS

J.J. Roberts, R.J. Knox, M.F. Pera, F. Friedlos and D.A. Lydall

INTRODUCTION

Evidence that the cytotoxicity of platinum compounds is due to their reactions with DNA came from studies of these reactions with the DNA of cultured cells and from studies of the responses of prokaryotic and eukaryotic cells to modifications of their DNA by these agents (1,2). However while such data adequately explain the cytotoxicity of platinum compounds in vitro, evidence implicating reactions with DNA in the anti-tumour effects these compounds is limited. Important questions therefore are whether cells differ in their responses to platinum compounds and whether such differences are a function of the extent of drug uptake and binding of platinum to macromolecules? Alternatively, do cells differ in their sensitivities to a given amount of DNA bound platinum and, if so, is this due to differences in their abilities to remove platinum adducts from their DNA by various excision repair processes or to differences in their abilities to tolerate lesions in their DNA when such damaged DNA undergoes replication? Furthermore, do analogous differences in the sensitivities of tumour cells in vivo account for the clinical responsiveness of some

human tumours to cis-diamminedichloroplatinum(II) (Cisplatin) or cis-diammine-(1,1-cyclobutanecarboxylato)platinum(II) (carboplatin)? To begin to answer these questions we have measured the binding of platinum to cellular DNA for given effects on cell survival for a number of rodent and human cell lines and related these values to those found for the binding of platinum to the DNA of cells isolated either from tumours in rodents or from patients receiving Cisplatin or carboplatin chemotherapy.

Measurements of the levels of one specific reaction of platinum compounds with DNA namely an interstrand crosslink, and studies of its repair in cells sensitive or resistant to Cisplatin has permitted an assessment of its likely importance in inducing toxic effects relative to that of other lesions such as an intrastrand crosslink.

MATERIALS AND METHODS

Cell lines and treatment with platinum compounds.

The conditions for growing HeLa and Chinese hamster cells (3) and Walker rat carcinoma cells (4) in culture and treating them with platinum compounds have been described previously. The establishment and cell biological properties (5) of the human germ cell lines and their treatment with Cisplatin (6) are described elsewhere.

Determination of platinum bound to DNA.

DNA was extracted from aliquots of between 5×10^7 and 1×10^8 cultured cells, from portions of excised rodent or human

tumours or from human ascites cells obtained from patients receiving platinum chemotherapy by a modification of the Kirby phenol method and hydrolysed in concentrated HCl. The nucleic acid base content was determined by uv spectroscopy and the platinum content of the hydrolysate was measured by flameless atomic absorption spectrometry (7).

Determination of DNA interstrand crosslinking by platinum compounds.

Several different methods were used for measuring crosslinks between strands of DNA isolated from cultured cells (7,8) and all gave values for crosslinking in good agreement with each other.

Plasmids.

pSV2gpt (9) was produced by conventional plasmid amplification techniques as previously described (10) then platinated and transfected into Walker sensitive or resistant cells all as previously described(11).

RESULTS AND DISCUSSION

Reactions of platinum compounds with the DNA of cultured cells.

Earlier studies of the reactions of platinum compounds with the DNA of cultured cells led to the postulate that only in the case of DNA molecules, as compared with RNA or protein molecules, is there sufficient reaction to account for the observed toxicity (12). Thus at a dose of Cisplatin (D_0) that reduces cell survival from f to 0.37f on the exponential portion of a semi-logarithmic survival curve, ie. the dose theoretically required to produce one

inactivating event, the binding to various macromolecules could be obtained as a value B_0 in nmoles Pt /g. These platinum binding values were essentially the same for the three macromolecules examined when expressed on the basis of weight. When however they were expressed in terms of their molecular weights it could be calculated that only in the case of DNA was there more than one reaction per molecule at this inactivating dose of drug presuming no preferencial binding to a particular sub-species of these molecules.

Table 1. Binding of Platinum to DNA of Various Cell Types at D_o Dose of Cisplatin

Normal human fibroblast (FL/G)	7.5
Normal human fibroblast (FL/A)	7.4
V79 Chinese hamster cells	8.5
Walker rat carcinoma cell (cisplatin resistant)	10.0

Over a period of many years we have measured the binding of platinum to the DNA of cells treated with a variety of doses of Cisplatin and the cell killing effects of such treatments. From these data we have established the relationship between cell survival and platinum bound to DNA and hence their B_0 values. In every case it could be argued, as above, that DNA is the most likely target for these cells. However it was also apparent that cells could

differ markedly in their sensitivity to DNA damage as assessed by their different B_0 values. Thus for a number of human and rodent cell lines the B_0 values were around 5-10 nmoles /g DNA (table 1). On the other hand fibroblasts derived from individuals with the genetic disorders Xeroderma pigmentosum (13) or Fanconi's anaemia (6) and cells of the Walker rat carcinoma (4) show unusual sensitivity to Cisplatin-induced DNA damage with B_0 values considerably lower than those for the above cells (table 2).

Table 2. Binding of Platinum to DNA of Human Embryonal Carcinoma Cells and Other 'Sensitive' Cell Types at D_o Dose of Cisplatin

Cell Line	B_o (nmoles Pt/g DNA)
GCT27) Human	4.0
GCT35) Embryonal	3.4
GCT46) Carcinoma	1.3
F9: Mouse Embryonal Carcinoma	3.8
Fanconi's anemia fibroblast strain	1.4
Xeroderma pigmentosum fibroblast (XP12BE)	1.8
Walker rat carcinoma cell (wild-type)	0.8

Table 2 shows that cells derived from some human embryonal carcinomas (6) are also more sensitive to the effects of DNA bound platinum than most cells examined although less

sensitive than the above genetically abnormal cells. It may be noted that a line of so-called resistant Walker tumour cells derived from the survivors of repeatedly treated sensitive Walker tumour cells had a sensitivity to platinum that was comparable to that of most other cells in culture (4)(table 1).

Reactions of Platinum compounds with the DNA of normal or tumour cells in vivo

That the extent of reaction of platinum compounds with the DNA of cells in vivo attains levels comparable those observed in cells in culture has now been clearly demonstrated in a number of studies. The relationship between the binding of platinum to the DNA of normal or tumour mouse cells (14) or human tumour cells growing in a nude mouse and their subsequent survival is shown in table 3.

Table 3. Relationship Between Binding of Cisplatin to DNA and Survival of Normal and Tumour Cells in vivo

	Dose of Cisplatin	Survival (% control)	Binding to DNA (nmoles/g)
Mouse bone marrow	10mg/kg	20 (spleen colonies)	10
Mouse B16	4mg/kg	80 (lung colonies)	10
Human pancreatic tumour xenograft	10mg/kg	10 (in vitro)	6

Again, the amounts of platinum bound to the DNA of Yoshida tumour cells obtained from rats treated with three different

platinum compounds were comparable to those observed to the DNA of cells in culture when treated with what were considered to be equivalent toxic doses of these compounds (table 4). It is apparent in all these studies that very comparable levels of reaction of platinum with cellular DNA (values of around a few nmoles Pt/g DNA) were observed for comparable effects on cell survival.

Table 4. Binding of Platinum Compounds to the DNA of Tumour Cells *in vivo* and Chinese hamster cells *in vitro*

	ADJ/PC6 tumour		Chinese hamster V79 cells	
Compound	ID_{90} (mg/kg)	Binding to tumour DNA (max)(nmoles/g)	D_o (uM)	Binding to DNA (nmoles/g)
Cisplatin	1.6	3.5	15	8.5
Carboplatin	14.5	1.8	120	3.0
CHIP	4.2	2.0	48	2.5

We have now measured the amount of platinum bound to DNA isolated from the cells of patients who had received either Cisplatin or carboplatin chemotherapy. As seen in table 5 the levels of platinum on the DNA of cells obtained from the ascitic fluid removed from patients with ovarian carcinoma at various times after they had received different doses of Cisplatin were all remarkably similar and were similar to the values found for the binding of platinum to the DNA of cultured cells . Similarly, the levels of binding of platinum to the DNA of cells obtained from patients who had

received carboplatin chemotherapy were comparable to those found to the DNA of cells in culture or cells from rodents treated with this agent (10).

Table 5. Relationship Between Dose of Cisplatin and Binding of Platinum to Human DNA in vivo

Cell type	Dose of Cisplatin (mg/m^2)	Time of Biopsy (hr)	Binding to DNA (nmoles/g)
Ovarian ascites cells	$100mg/m^2$	62 (after last dose)	11.2
	$30mg/m^2$	24	10.3
ovarian carcinoma - pleural effusion cells	$50mg/m^2$	28	28.8

These observations therefore support the notion that the anti-tumour effects of platinum compounds like their cytotoxic effects result from interactions with DNA. However it is also apparent that the amount of platinum bound to the DNA of human tumour cells would not be expected to produce much cell killing unless the tumour cells had a sensitivity to DNA-bound platinum that approached that of the sensitive cells listed in table 2.

Nature of DNA lesion leading to cytotoxicity.

It has been a matter of contention for a number of years as to which of the many known reactions that platinum drugs undergo with DNA is responsible for their toxic effects on cells. Thus the different chemical properties of Cisplatin

and its trans isomer (transplatin) have been used to argue the likely importance of, on the one hand, intrastrand crosslinks between adjacent bases on the same strand of DNA and, on the other hand, interstrand crosslinks between bases on opposing strands; while both types of reaction are carried out by the active cis compound the inactive trans compound cannot crosslink neighbouring bases in DNA and is less efficient than the cis compound at forming interstrand crosslinks in DNA. However the formation of equal numbers of DNA interstrand crosslinks at equitoxic doses of the cis and trans compounds, albeit with very different levels of overall DNA reaction, argues strongly in favour of a toxic role for this reaction by platinum compounds (see 1 and 2 for refs.)

An alternative approach to this problem therefore has been to compare two cell lines which differ markedly in their response to Cisplatin. As discussed above Walker tumour cells are uniquely sensitive to Cisplatin while a derived cell line has the same sensitivity as a number of other normal or tumour cells. The two Walker cell lines exhibit the same relative difference in sensitivity not only to a number of other platinum compounds including transplatin and carboplatin but also to many other cytotoxic agents which are known to be capable of reacting difunctionally with DNA. A common feature of these compounds is their ability to form DNA interstrand crosslinks and in the case of three such compounds, sulphur mustard, Cisplatin and 2,4-dinitro-5-

aziridinyl benzamide (CB 1954) (which is converted by metabolism to a difunctional agent) we have shown that very similar levels of interstrand crosslinking are produced by equitoxic doses of these compounds (14). Although it is likely that any compound that is able to form DNA interstrand crosslinks will probably also be capable of forming intrastrand crosslinks in DNA the ratio of inter to intrastrand crosslinks would be expected to be very different for these various compounds. Hence the similar relative difference in effects on Walker sensitive and resistant cells shown by all these agents is compelling evidence that an ability to form interstrand crosslinks is the common mechanism by which they induce cytotoxicity in these cells.

Interestingly, it was found that UV irradiation, which in effect produces crosslinks between adjacent bases on the same strand of DNA (thymine dimers), but does not produce interstrand crosslinks, was equally toxic to both sensitive and resistant Walker cells.

A platinum-induced crosslink between strands of DNA is known to be removed from DNA presumably by an, as yet undefined, repair mechanism(s). It might be that the sensitive Walker cell, or, indeed, any other sensitive cell, is defective in its ability to repair such a lesion in DNA. We have therefore attempted to identify the lesion(s) responsible for the toxic effects of Cisplatin in cultured cells by comparing the removal of platinum-induced damage in DNA in cells that differ in their sensitivity to Cisplatin.

Extensive studies of the loss of platinum adducts from the DNA of Walker sensitive and Walker resistant cells have however failed to reveal any differences in DNA repair adequate to account for the large difference in cytotoxic response to Cisplatin. Thus cells of the Cisplatin-sensitive Walker tumour and from the derived resistant subline were equally capable of carrying out the first step of excision repair of interstrand crosslinks as judged from the kinetics of loss of crosslinked DNA in cells treated with Cisplatin (4). In addition both cell types excised total DNA platinum adducts (which probably approximates to the intrastrand adduct since this forms about 60% of the total reaction products (16)) at comparable rates.

The above studies did not exclude the possibility that a later step(s) in the repair of interstrand crosslinks was defective in sensitive cells or that the fidelity of repair of platinum adducts could differ in the sensitive and resistant cells. Accordingly, we have examined the capacity of a cell to express the activity of a bacterial gene incorporated into a plasmid that had been damaged by Cisplatin prior to transfection into a recipient cell. It was found that sensitive and resistant Walker tumour cells were equally capable of repairing damage to the gpt gene (which provided a dominant marker conferring resistance to mycophenolic acid) contained in the plasmid pSV2gpt. Similarly, damage induced by chloro(diethylenetriamine)platinum(II) chloride (Pt(dien)),

a monofunctionally-reacting platinum compound was repaired equally in both cell lines although the gene could clearly tolerate many more platinum adducts introduced by the Pt(dien) than by the difunctional Cisplatin (fig 1). These inactivation curves, demonstrating the increased ability of Cisplatin compared to the Pt(dien) to inactivate a biologically active exogenous DNA molecule like the plasmid, therefore reflect the increased cytotoxicity towards mammalian cells of the difunctional as opposed to a monofunctional compound (11).

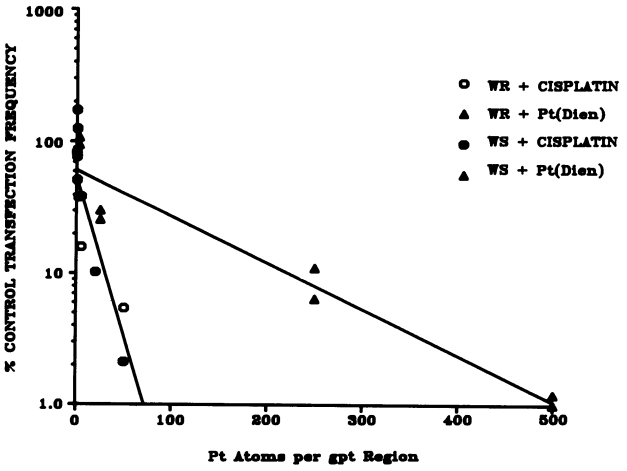

Fig 1. The effect of Pt adducts formed on pSV2gpt by Cisplatin (circles) or Pt(dien) (triangles) on the subsequent expression of XGPRT in either Walker sensitive (closed symbols) or Walker resistant (open symbols) cells.

It can be seen in fig 1 that about 20 platinum adducts in the gpt region of the plasmid were sufficient to reduce its survival to 10% that of the control untreated plasmid. Since other studies (17) have established that the

proportion of total platinations involved in the formation of an interstrand crosslink in platinated DNA is around 2% it follows that the gpt gene is inactivated by reactions other than an interstrand crosslink. Further since an intrastrand crosslink between adjacent purines is the predominant adduct in DNA platinated with Cisplatin it is likely that it is this lesion which is responsible for the inactivation of the gpt gene. It follows therefore that while this transfection system could be used to distinguish between the repair of damage induced by different platinum compounds such as the Pt(dien) and Cisplatin and to demonstratate equal repair of intrastrand crosslinks in the two cell lines it could not be used to detect a possible difference between sensitive and resistant Walker cells in the repair of an interstrand crosslink. In order to use this particular transfection system to detect the fidelity of repair of interstrand crosslinks in sensitive or resistant cells it will be necessary to use platinated plasmid that has been selectively enriched for interstrand crosslinks. Currently therefore it has not proved possible to detect, by means of these DNA repair studies, any defect in the sensitive Walker cells in the excision repair of either inter or intrastrand crosslinks such as would indicate the relative importance of one or other of these lesions in inducing toxic effects in cells. It should however be noted that the above transfection studies do confirm, in a mammalian cell system, what has been known for some time from bacterial studies (18), namely that a

platinum adduct on one strand of DNA such as an intrastrand crosslink is perfectly capable of inactivating a biologically active DNA molecule like a plasmid or a bacteriophage.

Genetic and biochemical studies in bacteria indicate that recombination events, as well as excision repair, are required for the repair of DNA interstrand crosslinks. Moreover the failure of sensitive cells in contrast to resistant cells to recover from the sulphur mustard-induced depression of DNA synthesis (19) could be a manifestation of their failure to complete the final steps in the repair of crosslinks and ones which could involve recombination. However both the sensitive and resistant Walker cells were shown to be equally capable of both ligation of endonuclease-induced double strand breaks in their DNA (although the efficiency varied with the actual site of the break) and homologous recombination of pSV2gpt fragments containing overlapping sequences of the gpt gene (11). The abilities of the two cell types to carry out recombination of integrated (as distinct from the above exogenous) gene fragments was examined using the plasmids pDR1 and pIR1 (kindly supplied by Drs. Subramani and Rubnitz (20)) which contain two truncated (and therefore nonfunctional) non tandem but overlapping, segments of the neo gene separated by a functional transcription unit coding for the gpt gene. This plasmid was transfected into the sensitive or resistant Walker cells using the gpt gene to select transfectants.

Subsequently recombination of the integrated defective neo gene segment was assayed by the appearance of G418 resistant cells. Recombination between the homologous regions of the segments was confirmed by Southern blot analysis. The results indicate a wide clonal variation in the recombination frequencies of the transfectants examined, but no significant differences in the recombination frequencies observed in sensitive as compared with resistant Walker cells. These various studies therefore did not reveal any inherent defect in the ability of the sensitive Walker cell to carry out recombination per se. On the other hand they do not exclude the possibility that sensitive Walker cells are defective in the proposed recombinational step in the repair of DNA interstrand crosslinks following treatment with Cisplatin.

In summary, therefore, our quantitative studies on the binding of platinum to the DNA of both cultured cells and rodent and human cells in vivo have further confirmed the likely importance of reactions with cellular DNA in inducing cytotoxicity to cells in culture and anti tumour effects in vivo. In addition these DNA binding data suggest that those tumours which do respond to platinum chemotherapy such as embryonal carcinoma may do so because they are composed of cells inherently sensitive to DNA bound platinum. The cross sensitivity of a line of cells to a variety of difunctional agents all capable of inducing interstrand crosslinks into DNA strongly emphasizes a cytotoxic role for this particular reaction. Finally, the complexities of the mechanism for

repairing interstrand crosslinks in DNA probably accounts for our failure to establish the importance of interstrand crosslinks by demonstrating their defective repair only in cells sensitive to Cisplatin.

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IN VIVO CIS-DIAMMINEDICHLOROPLATINUM(II)-DNA ADDUCT FORMATION AND REMOVAL AS MEASURED WITH IMMUNOCHEMICAL TECHNIQUES

A.M.J. Fichtinger-Schepman, F.J. Dijt, W.H. De Jong, A.T. Van Oosterom and F. Berends

INTRODUCTION

The cytostatic agent cis-diamminedichloroplatinum(II) (cisplatin) can react with DNA and RNA as well as with proteins. It is generally assumed that the antineoplastic activity results from the interaction with DNA inside the cell nucleus. Therefore, studies on the induction and repair of cisplatin-DNA adducts (e.g. in cultured cells) have been and are being performed to get insight into the working mechanism of cisplatin. In Fig.1 the various types of reaction products are depicted. Despite a multitude of investigations in the last 10 years, it is still unknown which of the adducts is responsible for the antitumor action or for the

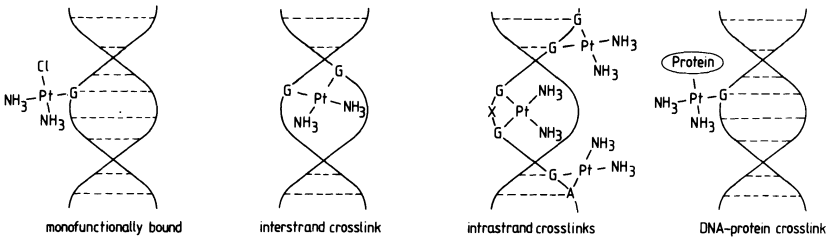

Fig.1. The known cisplatin-DNA adducts.

(undesired) side effects of the drug. An important bottleneck in these studies was the lack of analytical methods for the quantitative detection of the DNA adducts at a level of sensitivity and selectivity that would permit their determination in DNA of mildly exposed cells or tissues.

Our approach to overcome this problem has been based on the development of immunochemical detection methods, in combination with chromatographic separation procedures. Presently, we are able to assay four different cisplatin adducts in small samples of enzymatically degraded DNA, at the fmol level. The principles of our method and the results obtained with DNA from various sources, including cells from treated human cancer patients, will be presented in this paper.

"FIXATION" OF CISPLATIN-DNA MONOADDUCTS

As can be seen in Fig.1, in most types of adducts cisplatin has reacted bifunctionally. However, especially after short incubation periods, also monofunctionally-bound cisplatin adducts are present in the DNA. In these adducts, the remaining coordination site of cisplatin is still reactive. Its through-reaction with other groups in DNA, or with other molecules, after sampling of the exposed materials would distort the spectrum of DNA adducts determined subsequently. To prevent the uncontroled formation of additional crosslinks, the free coordination site has to be blocked. In 1984 we demonstrated (1) that this could be realized by the introduction of an NH_3 group, by an overnight incubation with an ammonium salt, i.e. NH_4HCO_3.

In 1986, however, Eastman (2) reported a different inactivation procedure, with thiourea, which he claimed to be superior to our method. This claim was based on the observation that much higher proportions monofunctional adducts were found with his method than we had obtained with NH_4HCO_3, and on the consideration that the inactivation with thiourea proceeds much faster (10 min at 23°C) which reduces the chance for the monoadduct to react with groups in DNA. Eastman reported that after a short treatment of DNA *in vitro* with the cisplatin analog *cis*-dichloro-(ethylenediamine)platinum(II) (15min; 37°C), 42% of

DNA-bound platinum was present as the monoadduct. In contrast, in our kinetic studies on the reaction of cisplatin with DNA - with NH_4HCO_3 as the inactivator (3) - the monofunctional adduct to guanine represented not more than 15% of total platination (after 30 min of incubation at 37°C, decreasing to 7% after 5 hr; lower percentages were found at 50°C).

In view of this discrepancy, and to check the reliability of the procedure used so far in our experiments, recently we extended the work on the use of NH_4HCO_3 as the "fixation" agent. In this study we included dGMP as a competitor for the ammonium compound, to mimic the potential reaction of the monoadduct with free guanines in DNA, the nucleobase with the highest reactivity towards cisplatin.

Incubation of dGMP with cisplatin in water for 25 hr at 37°C

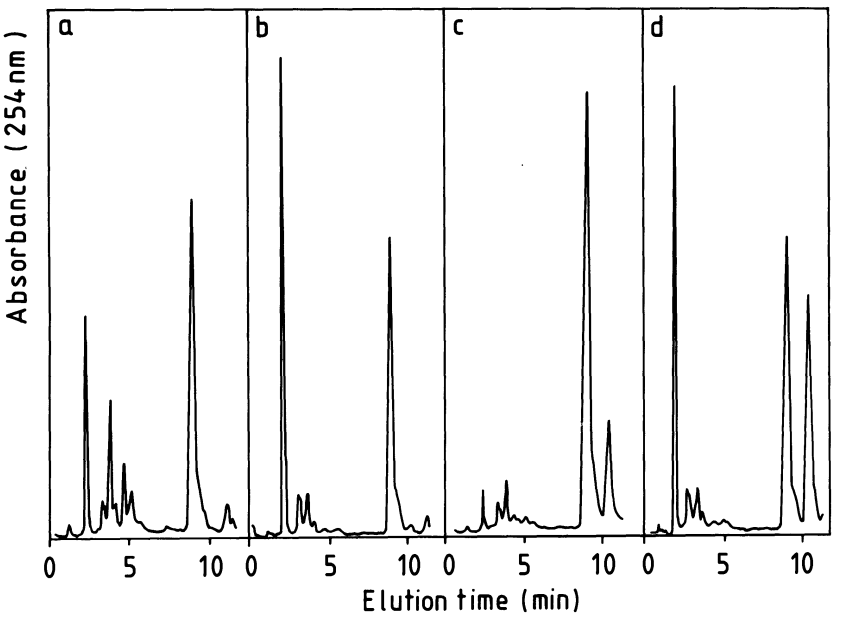

Fig.2. Anion-exchange chromatography (FPLC; Mono Q column, Pharmacia) of mixtures of dGMP and cisplatin incubated for 25 hr at 37°C, followed by an overnight incubation at 37°C with an equal volume of a: water; b: 0.2 M NH_4HCO_3; c: 1.0 mM dGMP and d: 1.0 mM dGMP + 0.2 M NH_4HCO_3 (see text). The main peaks eluted at about 2, 8 and 10 min consisted of Pt-G, G-Pt-G and dGMP, respectively. Most of the small peaks in panel a represent cisplatin monofunctionally bound to dGMP [cis-$Pt(NH_3)_2$(dGMP)X]. The peaks were detected by their UV absorbance (254 nm).

(dGMP 0.55 mM; cisplatin 0.5 mM) yielded a mixture of the monoadduct cis-$Pt(NH_3)_2$(dGMP)X (X = Cl, H_2O or OH) and the bifunctional adduct cis-$Pt(NH_3)_2(dGMP)_2$ [G-Pt-G]. The reaction mixture was concentrated to one quarter of its volume. Incubations were then continued overnight at 37°C after addition of an equal volume of a) water, b) 0.2 M NH_4HCO_3 (final concentration ratio salt : Pt = 100 : 1), c) 1.0 mM dGMP, or d) 0.2 M NH_4HCO_3 together with 1.0 mM dGMP. The mixtures were analyzed by anion-exchange chromatography (see Fig.2).

After incubation in the presence of NH_4HCO_3, the monoadducts had reacted to a compound with the structure $Pt(NH_3)_3$dGMP [Pt-G], as can be seen by comparing Figs. 2a and 2b. Fig. 2c shows that dGMP, in the absence of NH_4HCO_3, reacted with the monoadducts to give G-Pt-G. However, when both dGMP and NH_4HCO_3 were present during the second incubation, no significant conversion of the monoadducts into G-Pt-G occurred, as the formation of Pt-G was not different from that in the absence of dGMP (compare Figs. 2d and 2b). On the other hand, an increase of the dGMP concentration to 2mM resulted in some formation of G-Pt-G in the presence of 0.1 M NH_4HCO_3 (not shown).

In a second experiment, once more the reaction of DNA with cisplatin was studied, but now special attention was given to the shorter incubation periods. Fixation again was with 0.1 M NH_4HCO_3 (overnight dialysis). The resulting DNA samples were analysed for cisplatin adducts (see next section). The results shown in Table I illustrate that also with NH_4HCO_3 fixation high percentages of monoadducts can be found (43% after 15 min cisplatin treatment, decreasing to 14% after 60 min). These results demonstrate the efficacy of the NH_4HCO_3 procedure for blocking the reactive coordination site in monofunctionally-reacted cisplatin.

CISPLATIN-DNA ADDUCT MEASUREMENTS

The methods to detect and quantitate the different cisplatin adducts formed in DNA have been developed with salmon sperm DNA treated in vitro with cisplatin (3,4). After cisplatin treatment followed by the NH_4HCO_3 "fixation procedure" (see above), DNA is

Table I. Cisplatin treatment of salmon sperm DNA at 37°C.

DNA (0.5 mg/ml) was incubated with cisplatin (10 µg/ml) in 0.01 M phosphate buffer, pH 7.2, at 37°C for the periods indicated. The reaction was stopped by addition of NH_4HCO_3 to a final concentration of 0.1 M, followed by overnight dialysis against 0.1 M NH_4HCO_3 at 37°C. Then, DNA was digested nucleolytically and analysed for the 4 Pt-containing products mentioned, by ion-exchange column chromatography (see Fig. 2). The Pt-content of the fractions was assayed with AAS.

Incubation time (min)	R_b [1]	% of total DNA platination [2]				
		Pt-G	Pt-AG	Pt-GG	G-Pt-G	incompletely digested DNA [3]
15	0.4	43	6	49	2	0
30	1.0	21	8	65	6	0
45	1.6	16	9	68	7	0
60	2.5	14	12	64	9	0
30 [4]	1.8	15	12	57	6	10
120 [4]	9.6	12	14	65	5	4

1 Number of Pt-atoms bound per 10^3 nucleotides in the DNA sample;
2 See text for definition of abbreviations;
3 Eluted at high salt;
4 Data from earlier experiments (3).

digested with the deoxyribonucleases DNAseI and P1 nuclease to yield unmodified mononucleotides and Pt-containing (oligo)-nucleotides. The latter comprise (cf. Fig.1):

- $Pt(NH_3)_3dGMP$ [Pt-G], derived from cisplatin monofunctionally bound to guanine,
- cis-$Pt(NH_3)_2d(pApG)$ [Pt-AG], derived from intrastrand crosslinks of cisplatin on neighboring nucleobases adenine and guanine,
- cis-$Pt(NH_3)_2d(pGpG)$ [Pt-GG], derived from intrastrand crosslinks on base sequences pGpG,
- cis-$Pt(NH_3)_2(dGMP)_2$ [G-Pt-G], the digestion product from intrastrand crosslinks on two non-neighboring guanines $[pG(pX)_npG]$ and/or from interstrand crosslinks on guanines.

In all these cisplatin-DNA digestion products the platinum is bound

to the N7 atoms of guanine or adenine. The identity of the products was established by proton nuclear magnetic resonance measurements (3,4).

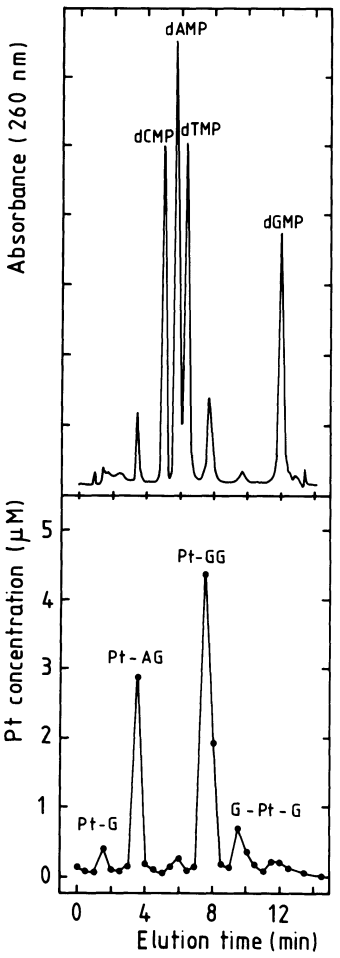

Fig.3. Anion-exchange chromatography of digested cisplatin-DNA. Salmon sperm DNA (0.5 mg/ml) was reacted with cisplatin (25 μg/ml; 5hr; 37°C), treated with NH_4HCO_3 and digested with nucleases. The digest was fractionated by FPLC on the Mono Q column. The unmodified mononucleotides dCMP, dAMP, dTMP and dGMP were detected by their UV absorbance (260 nm; upper panel). The Pt-containing products were measured with AAS (lower panel).

To quantitate these Pt-containing (oligo)nucleotides, the DNA digestion products are separated on an anion-exchange column at pH 8.8, and the amounts of platinum are established in the collected column fractions with atomic absorption spectroscopy (AAS). Fig.3 shows the results of this method applied on DNA digested after a prolonged incubation (5 hr) with cisplatin (3). Pt-GG is the main product followed by Pt-AG, while G-Pt-G and Pt-G represent minor adducts. Table I gives data for the various adducts after short incubation periods; in these samples the monoadducts (Pt-G) occupy a much more dominant position. The total of the four Pt products determined in the newly analysed samples comprised all the Pt bound to the DNA's; no Pt-containing material was eluted from the column with high salt.

These results indicate that in in vitro cisplatin-treated DNA, eventually the Pt-GG crosslinks are by far the main adducts. Initially, however, the monofunctional reaction products with guanine represent a large proportion of the platination sites. The results suggest that in general the Pt-G adduct will be the initial product, which is fairly rapidly converted into intra- or interstrand crosslinks.

IMMUNOCHEMICAL QUANTITATION OF THE CISPLATIN-DNA DIGESTION PRODUCTS

The platination levels in DNA treated with biologically relevant cisplatin dosages are often very low. In these cases the AAS is not sensitive enough. To become able to measure the small amounts of adducts present, we elicited antibodies against cisplatin-containing nucleotides covalently coupled to immunogenic carrier proteins. With three different rabbit antisera, fmol amounts of all four cisplatin-DNA digestion products can now be quantitated (5,6) in a competitive enzyme-linked immunosorbent assay (ELISA). In this assay the cisplatin-DNA digestion products, present in the fractions of the Mono Q column, are used as inhibitors of antibody-binding to immobilized antigen, i.e. cisplatin-treated DNA adsorbed to the wells of the microtiter plate. The total detection procedure has been developed to be applicable on DNA isolated from cisplatin-treated mammalian cells. Recently, it has been successfully applied to detect cisplatin-DNA adducts in nucleated blood cells of cancer patients receiving cisplatin chemotherapy.

CISPLATIN-DNA ADDUCTS IN CULTURED MAMMALIAN CELLS

For the determination of the adducts, first the cisplatin-treated cells are lysed by an overnight incubation with sodium dodecyl sulfate, in the presence of NH_4HCO_3 necessary for the fixation of the monofunctional adducts, and of proteinase K (6). The latter enzyme will inactivate the cellular enzymes that break down DNA, which would interfere with its isolation, and digest proteins possibly crosslinked to DNA by cisplatin (see Fig.1) and thus reduce the protein part of these crosslinks to one or a few aminoacids. DNA is isolated from the cell lysates by phenol and

chloroform/isoamyl alcohol extractions, RNAse treatment and alcohol precipitations. Then, the isolated DNA is treated with nucleases and the digest is chromatographed, followed by (immunochemical) quantitation of the Pt-products. Until now, we do not know in which eluate fraction(s) of the column the digestion products of the DNA-protein crosslinks are to be detected. From other studies, e.g. Plooy et al. (7,8), it is known that in cultured cells this type of adduct comprises only approximately 1% of the total platination in DNA.

In all cell types tested so far, cisplatin treatment appeared to induce the same spectrum of DNA adducts as was found in DNA after in vitro cisplatin treatment. In all cases the Pt-GG adduct was the main adduct induced, representing 50-75% of total platination.

To study the influence of DNA-repair systems, the induction and repair of DNA adducts in various human fibroblast lines were compared. This comparison included cells from a "normal" person (82MB2), from a Fanconi's anemia (FA) patient and from a xeroderma pigmentosum (XP) patient (complementation group A). The FA and XP cells, which are extremely sensitive to cisplatin with regard to survival (8), have DNA repair defects. FA cells are assumed to have an insufficient capacity to repair DNA interstrand crosslinks, whereas XP cells are deficient in excision repair.

Upon identical treatments of these cells with cisplatin, FA cells differed from the other fibroblasts by showing about 50% more induction of DNA adducts per amount of DNA.

During a post-treatment incubation, performed to compare repair of DNA adducts, differences were observed with regard to the removal of Pt-GG adducts. Here, the XP cells were exceptional. Fig. 4 shows the relative amounts of the main adduct Pt-GG present in the DNA of the three cell lines directly after the cisplatin treatment (100%) and after 4.5-hr and 22-hr post-incubation periods. The data clearly show that in the normal as well as FA cells, a large fraction of the adducts disappears rapidly in the first hours after the cisplatin treatment, whereas in XP cells hardly any removal of Pt-GG was observed. In the subsequent period (4.5 - 22 hr) little

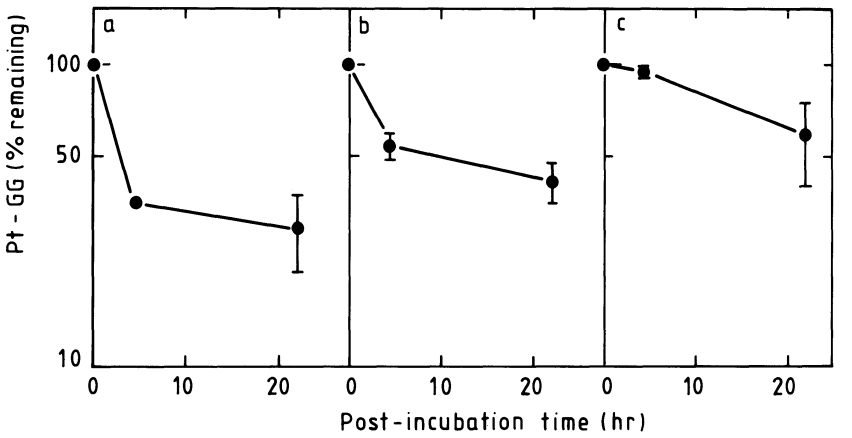

Fig. 4. Percentages of the main adduct Pt-GG remaining in the DNAs of cultured human fibroblasts after post-incubation periods following the cisplatin treatment. The adducts were measured with antibodies in the competitive ELISA. a: normal cells; b: FA cells and c: XP cells.

additional removal occurred in normal and FA cells, whereas XP showed some removal over that period. In total, normal cells appear to remove a larger proportion of the adducts than FA cells, but not more in absolute numbers.

The results of the XP cells suggest that deficient DNA-excision repair in human cells results in an enhanced cytotoxicity of cisplatin. The reason for the increased sensitivity of FA cells for cisplatin is not so obvious. This may partly be attributed to the increased amounts of DNA adducts formed and the smaller proportion of adducts removed in FA compared with the normal cells, resulting in the presence of about the same number of adducts in the DNA of FA cells as in XP cells after 22 hr post-incubation. In view of the results of Plooy et al. (8), however, the persistence of the interstrand crosslinks observed by these authors in cisplatin-exposed FA fibroblasts can be thought to give an important contribution to the high sensivity of these mutant cells to cisplatin.

CISPLATIN-DNA ADDUCTS FORMED IN VIVO; HUMAN BLOOD CELLS

After proper adaptation to the material under study, our

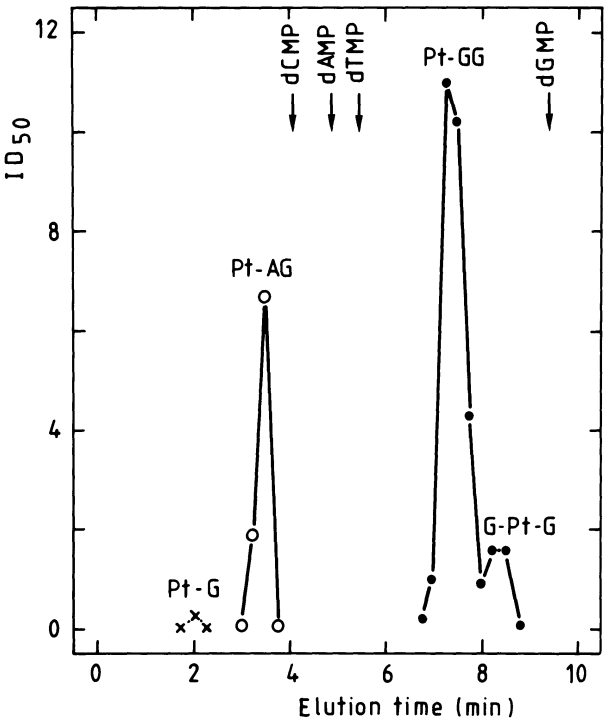

Fig.5. Cisplatin-DNA adducts in white blood cells of human cancer patients. The figure shows the immunochemical quantitation of the adducts in eluate fractions of the ion-exchange column used in the chromatography of DNA digests. On the ordinate is plotted to which extent a fraction has to be diluted to give 50% inhibition of the amount of antiserum used in the competitive ELISA (ID50). Three rabbit sera were used: x serum 3/43 elicited against Pt-G; o serum 3/65 elicited against Pt-AG; • serum WI01 raised against cis-$Pt(NH_3)_2$GuoGMP. For all 3 sera the amount of reference compound giving 50% inhibition is between 4-10 fmol (6).

determination of Pt-DNA adducts based on nucleolytic digestion, chromatographic separation and immunochemical detection can be applied also on DNA derived from cells or tissues that have been exposed in vivo. The first adaptation realized was the detection of cisplatin-DNA adducts in nucleated human blood cells. Both after in vitro exposure of human blood and with blood from cisplatin-treated cancer patients, the white blood cells (WBC) were shown to contain the same four adducts that had been identified in DNA exposed in vitro or in DNA from cisplatin-treated cultured fibroblasts. Fig.5 shows the results obtained with a blood sample of such a patient.

It demonstrates clearly that also in the WBC exposed in vivo Pt-GG and Pt-AG are the dominating adducts.

As we have discussed before (6,9), it may be fruitful to investigate the formation and removal of cisplatin adducts in the DNA of WBC of patients as an indicator for the interindividual differences in the efficacy of cisplatin as an antineoplastic agent. Poirier et al. (10) reported a relation between the amount of DNA adducts in the blood of patients, treated with cisplatin for testicular or ovarian cancer, and the response of their tumors to treatment. Furthermore, their data indicated that the DNA adducts in WBC were persistent for at least a few weeks.

In our studies on cisplatin-DNA adduct levels in WBC of cancer patients, interindividual differences were observed after the 3-hr cisplatin infusion period (6,9). Next, the persistence of the DNA adducts in WBC in vivo was studied. Fig. 6 shows the amount of the

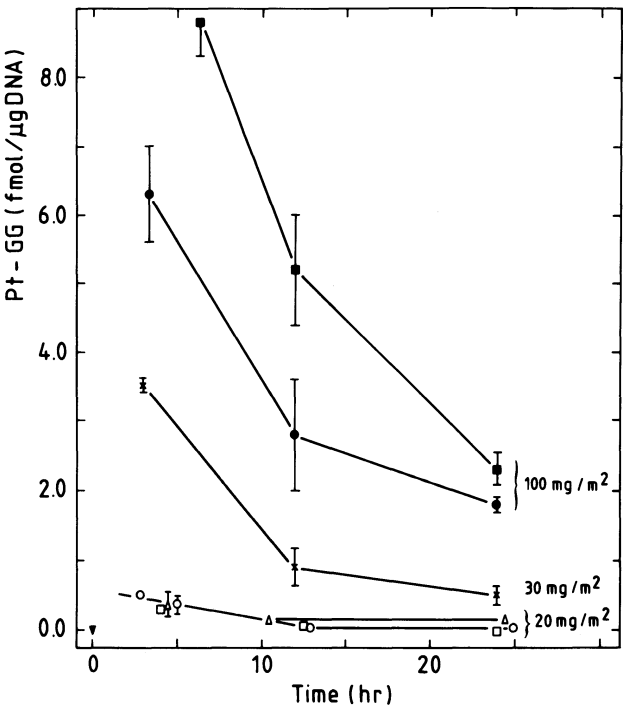

Fig.6. Induction and removal of the main cisplatin-DNA adduct in WBC of cancer patients (Pt-GG). They had been treated for the first time with cisplatin and received dosages of 20, 30 or 100 mg/m^2 body area.

main DNA adduct Pt-GG in blood samples collected at various time intervals after the first cisplatin treatment of six cancer patients. Evidently, in circulating WBC these adducts are removed rapidly.

In addition to efforts to develop a method for the prediction of the tumor response of cancer patients on the basis of the amount of DNA adducts in their WBC during the cisplatin chemotherapy, we try to develop a predictive in vitro assay: we compared the adduct formation after in vivo treatment of the patient with the induction in vitro during a 3-hr cisplatin treatment of blood collected from the patient just before the cisplatin infusion started (11). The results so far look promising, showing a correlation coefficient between both sets of data of 0.91. Experiments are ongoing to relate these data with the response of patients suffering from tumor types with a low cure rate.

CISPLATIN-DNA ADDUCTS FORMED IN VIVO; TUMOR-BEARING RATS

In the course of treatment of a cancer patient with cisplatin, frequently the tumor develops a certain resistance towards this drug. To study the mechanism underlying the development of such a resistance, we investigated an IgM immunocytoma tumor model in LOU/M rats (12). This transmissible solid tumor becomes resistant after repeated injections of the rat with cisplatin. We compared the induction of cisplatin-DNA adducts and their repair in the cisplatin-sensitive and in the resistant tumor, in rats that had developed the tumor after having been inoculated with cell suspensions.

The results indicated that 1 hr after an i.v. injection of 10 mg cisplatin/kg, about the same degree of DNA platination had occurred in the sensitive and the resistant tumor. However, at 24 hr after the injection, a larger proportion of the adducts had been removed in the resistant than in the sensitive tumor cells. Probably, an adaptation of the DNA repair induced by the cisplatin treatment of the original tumor cells had made the tumor less susceptible to cisplatin. This difference in cisplatin-DNA adduct repair capacity was observed between both tumor types, but not

between the WBC, or other tissues tested so far, of the rats bearing either the sensitive or the resistant tumor.

In these experiments, all tissues and organs tested showed the same spectrum of adducts as the exposed DNAs studied before. Large differences were observed, however, with regard to the degree of DNA platination. The total amounts of adducts present at 1 hr after the onset of the cisplatin treatment (10 mg/kg) ranged between 10 fmol and 400 fmol per µg of DNA; they could be ranked as follows: WBC < spleen < tumor < liver < kidney. The very high adduct level in the kidney samples even fell within the sensitivity range of AAS determinations. This allowed an independent check on the values assessed immunochemically: virtually identical results were obtained.

COMPLICATIONS IN THE IMMUNOCHEMICAL DETECTION

In our procedure of adduct detection, after its isolation DNA first has to be digested and chromatographed before the immunochemical assay can be performed. This procedure has the advantage that unmodified nucleotides and other materials that might interfere with the quantitative ELISA are removed before this assay is carried out, and that the four adducts can be determined separately, with a positive identification (specificity of the antiserum + elution position). Furthermore, in the competitive ELISA the compounds used for calibration can be identical to the Pt-products to be determined. Together, these factors greatly enhance the reliability of the results.

It is a rather laborious procedure, however, and many more determinations might be performed if the cisplatin-modified DNA itself could be used for the detection of the adducts. This form of detection is a prerequisite when the study of adducts in microscopic preparations of exposed tissues/cells is intended (9). For these applications, however, the antibodies should comply with strong requirements with regard to specificity and selectivity.

Recent findings demonstrate some of the problems that may arise when the competitive ELISA is used to quantify adducts in DNA directly. Our results obtained with WBC from cisplatin-treated patients indicated much higher levels of DNA platination than those

published by Poirier et al. (10) who applied this direct assay. A similar difference now emerges with regard to the adduct levels in cisplatin-exposed experimental animals: the values we obtained (see preceding section) are about 1000 times as high as those described by Reed et al. (13). In both publications (10,13) the same anti-cisplatin-DNA antiserum was used for the direct determination of adducts in isolated DNA (14). Since our kidney data could be confirmed by AAS measurements, it appears that the use of the anti-cisplatin-DNA antiserum may lead to erroneous results. The problems probably have to be attributed to the characteristics of this antiserum. It has been raised by immunizations with highly-platinated DNA, and recently it has become clear that it recognizes only poorly adducts in DNA with a low degree of cisplatin-binding (M.C. Poirier, personal communication). For the calibration of the competitive ELISA, however, DNA had been used with a rather high Pt-content, which was needed to allow AAS determinations. The phenomenon is in agreement with our experience (5) that an antiserum raised against a cisplatin-nucleotide hapten bound well to DNA only when it was highly platinated; indications were obtained that in DNA with low levels of platination the adducts are not too accessible for the antibody molecules.

CONCLUDING REMARKS

With the availability of immunochemical techniques to measure the various cisplatin-DNA adducts in all types of cells treated *in vitro* or *in vivo* at biologically relevant cisplatin dosages, the objective of gathering information about the working mechanism of cisplatin at the DNA level has become accessible. Knowledge about the adduct formation and repair may lead to the identification of the "antitumor lesion", give insight in the differences in cisplatin-susceptibility between the individual patients, between the various tumor types, but also between the cisplatin-sensitive tumor cell and its resistant daughter cells. Furthermore, data of the adduct-behaviour in various tissues and organs may provide information on the origin of the undesired effects of cisplatin and, thus, may help to find ways to overcome these. Especially when attempts are successful to obtain (monoclonal) antibodies that

allow the selective and very sensitive recognition of the adducts in DNA at the single-cell level, then the research aims described above will become attainable within the next years.

ACKNOWLEDGEMENTS

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OLIGONUCLEOTIDE MODELLING OF *CIS*-PLATINUM BINDING TO DNA

J.C. Chottard

INTRODUCTION

In the cell, DNA appears as the primary target of the aquated forms of the antitumor drug cis-diamminedichloroplatinum (II) (cis-DDP) (2,3). We wish to focus on two aspects of this interaction :

i. Several *in vitro* studies have shown that the main platinum adducts are the intrastrand N7,N7 d(GpG) and d(ApG) chelates of the cis-$Pt(NH_3)_2^{2+}$ moiety (4,6). They respectively account for about 60-65% and 20% of the platinum bound to salmon sperm DNA (5). These results show that platinum not only first binds to a guanine, as recently proved by the identification of monofunctional adducts (7) but also binds preferentially to a guanine located in a $(dG)_n$ sequence ($n \geq 2$). For salmon sperm DNA, having 20.5% G, a random distribution of equireactive guanines should lead to a maximum of 37% of d(GpG) adduct (5), or 43% if one takes into account the correcting factor of 1.16 found in the study of the nearest neighbor nucleotide patterns of thirty eukaryotic sequences (8).

ii. Several studies were devoted to the perturbation of the DNA secondary structure due to its platination. A decrease of the viscosity (9), an induction of S1-nuclease activity at r_b 0.025 (r_b = number of platinum atoms bound per nucleotide) (10, 11) a shortening and unwinding of platinated DNA (12,13) all revealed a distortion of the duplex structure. All the relevant data have been thoroughly analyzed in recent reviews (14-16) to which the reader is referred.

Using oligonucleotide models we have addressed the following two questions : why does cis-DDP selectively bind to guanines within

$(dG)_n$ sequences ? What is the exact deformation caused by the formation of a d(GpG).cis-$Pt(NH_3)_2$ adduct ? This paper will summarize our recent results obtained by this approach in relation to the determinant contributions from other groups.

SELECTIVITY OF THE FIRST PLATINUM BINDING

All the data from mononucleotide to DNA-studies show a kinetically favored platinum binding to the N7 atom of guanine (14,16). A 5'-phosphate group enhances the rate of G-N7 platination. A factor of 15 was found between 5'-GMP and G for their platination by cis-$[Pt(NH_3)_2(H_2O)_2]$ $(CF_3SO_3)_2$ (17). Whereas a competitive experiment between 5'-GMP and 5'-dGMP, reacting with aquated cis-DDP, revealed no difference between the two nucleotides (18), a kinetic study found a ten times faster first step for the deoxy-nucleotide (19). 5'-GMP and 5'-dGMP are platinated faster than their 3' counterparts (18,20). A phosphodiester group has a smaller influence than a terminal 5'-phosphate, still favoring N7 platination on the 3'-side. The ratios of the adducts formed by the reactions of GpG (21) and d(GAG) (22) with [PtCl(dien)] Cl, gave a factor of 2 in favor of the platination of the 3'-G compared to the 5'-G. We have chosen to study the influence of the adjacent base(s) on the platination of a guanine in three different situations : first within dinucleotides, second within single-stranded oligonucleotides, third within the corresponding double-stranded oligonucleotides, in order to see the contribution of the duplex structure. The aim is to get experimental data for each situation and to compare them with the results of a molecular mechanics analysis of the directing interactions which are present in the pentacoordinated intermediate of the first platination step. With a readily replaced leaving group like H_2O, the energy of the pentacoordinated intermediate should be close to that of the distorted trigonal-bipyramidal transition state of the substitution of an aqua ligand by GN7 (24).

Dinucleotides.

We have determined the rate constants (k_1) of the first platination step of the ribo-dinucleotides GpG, ApG, GpA, CpG and GpC, reacting with cis- $[Pt(NH_3)_2(H_2O)_2]$ $(NO_3)_2$ **1**, and

$[Pt(NH_3)_3(H_2O)](NO_3)_2$ **2.**

Table 1. k_1 rate constants ($M^{-1}.s^{-1}$) of the first platination of various dinucleotides by cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ **1** and $[Pt(NH_3)_3(H_2O)]^{2+}$ **2**, at pH 5.2 and 20°C.

	1	**2**
$k_{1(tot)}$GpG	8.5 ±1.5	0.92±0.05
$k_{1(3')}$GpG	5.7 ±1.0	0.61±0.02
$k_{1(5')}$GpG	2.8 ±0.5	0.31±0.02
$k_{1(3')}$ApG	1.7 ±0.1	0.40±0.02
$k_{1(5')}$GpA	0.8 ±0.1	0.31±0.02
$k_{1(3')}$CpG	2.2 ±0.1	0.52±0.05
$k_{1(5')}$GpC	0.95±0.1	0.44±0.05

For the deoxy-dinucleotides dGpG, dApG and dGpA reacting with **1** in the same conditions, one found $k_{1(tot)}$dGpG = 9±2, $k_{1(3')}$dApG = 1.5±0.2 and $k_{1(5')}$dGpA = 0.6±0.2 $M^{-1}.s^{-1}$. This indicates that there is no significant difference between oxy and deoxy compounds. Table 1 shows that the rate of platination by complex **1** is significantly larger than that by complex **2**, this being more pronounced for GpG (with a ratio close to 10) than for the other dinucleotides. By comparing the ratios of the k_1 rate constants, one can try to evaluate the influence of a neighboring guanine, compared to an adenine or a cytosine, on the N7 platination of a guanine. This is shown in Table 2.

Table 2. Influence of an adjacent 3'- or 5'-G, compared to an A or a C, on the k_1 rate constant of G platination by complexes **1** and **2**, and influence of the 5'-phosphodiester group on the 3'-G platination.

	relative to A		relative to C	
	1	**2**	**1**	**2**
3'-G	x(3.5±1.0)	x(1.0±0.1)	x(2.9±0.8)	x(0.7±0.1)
5'-G	x(3.3±0.8)	x(1.5±0.1)	x(2.6±0.6)	x(1.2±0.1)
5'-p	x(2.0±0.4)	x(1.3±0.1)	x(2.3±0.3)	x(1.2±0.2)

Taking into account the precision of the data one can draw several conclusions at the dinucleotide level.

a) For the platination by the diamminediaqua complex **1** : i) a 3'- or 5'-G, when compared to an A or C, favors by a factor of about 3 the platination of the other G ; ii) there seems to be a small difference between the influence of an adjacent A or C on G platination, in favor of C ; iii) a 5'-phosphodiester group increases the rate of platination of the 3'-G by a factor of about 2.

b) For the slower platination by the triamminemonoaqua complex **2** : i) only a 5'-G, when compared to an A or a C, seems to slightly favor the reaction of the other G ; ii) there is still a small rate increase for an adjacent C compared to an A. For a competition experiment between dApG and dCpG, reacting with [PtCl(dien)] Cl, the ratio of adducts gave a factor of 2 in favor of dCpG (23) ; iii) a 5'-phosphodiester group has a very small influence if any.

Thus, in the first platination step, on a guanine, interactions of the diaqua complex **1** with an adjacent guanine (3'- or 5'-) may play an important role.

We are running molecular mechanics calculations using the program AMBER (25) to compare the trigonal bipyramidal intermediate complexes of the first 3'-G platination of GpG and ApG by complexes **1** and **2**.

Table 3. Hydrogen bonds involved in the pentacoordinated intermediate of the 3'-G (G(2)) platination of GpG by cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ **1**.

Hydrogen bonds H---- X	Length (Å)	H/X interaction energy (kcal. mol^{-1})
eq OH_2 --- O5'	2.06	- 4.2
eq OH_2 --- OP	2.02	- 8.3
ax OH_2 --- N7-G(1)	1.92	- 6.4
ax OH_2 --- O6-G(1)	2.08	- 4.0
ax NH_3 --- OP	1.87	- 5.4
eq NH_3 --- O6-G(2)	2.38	- 3.3

Preliminary results indicate that the pentacoordinated intermediate of the 3'-G platination of GpG by **1** (Fig. 1) exhibits a stabilizing hydrogen bond between the axial H_2O ligand and the O6 of the 5'-G (G(1)) which cannot exist with the A(1) 6-NH_2 of ApG.

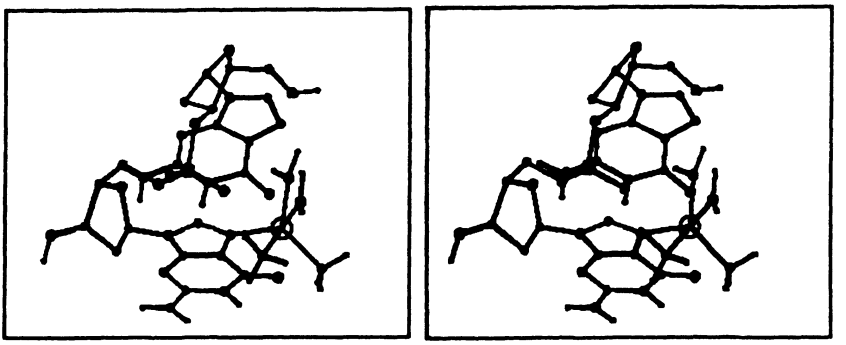

Fig. 1. Stereoview of the pentacoordinated intermediate complex of the 3'-G platination of GpG by cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$.

Similar calculations are in progress for the 5'-G platination of GpG and GpA. However the influence of a 3'-G on the platination of the 5'-G of a dinucleotide may be irrelevant to the case of larger oligonucleotides, because of the lack of contribution of a phosphodiester group on the 5'-side of the guanine to be platinated, and because of the larger mobility of the bases in the dinucleotide.

Hexanucleotides

We have compared the rates of platination of the GG and AG sequences within the complementary hexamers d(TGGCTA) **3** and d(TAGCCA) **4** and within their duplex **5**. From the UV melting profiles of **5**, we selected the following conditions to work with the actual duplex form : $[5] = 10^{-5}$M in 0.5M $NaNO_3$ (Tm=34°C) at 18°C. The reactions were run with a 1:1 ratio of cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ **1** (10^{-5}M) and either **3, 4, 5** in water or **3, 4, 5** in $NaNO_3$ 0.5M. The k_1 rate constants of platination of the single-strand hexamers were determined from HPLC monitoring of the reactions :

	$k_1(H_2O)$ ($M^{-1}.s^{-1}$)	$k_1(NaNO_3)$ ($M^{-1}.s^{-1}$)	$k_1(3)/k_1(4)$
d-TGGCTA **3**	150±25	5.8±0.3	4±0.5
d-TAGCCA **4**	-	1.4±0.1	

It is noteworthy that whereas **3** gives only the GG chelate, **4** gives the AG chelate and the bis(**4**) complex : cis-$[Pt(NH_3)_2\{TAGCCA\text{-}\underline{N7}(3)\}_2]^{2+}$ (Fig. 2a,b).

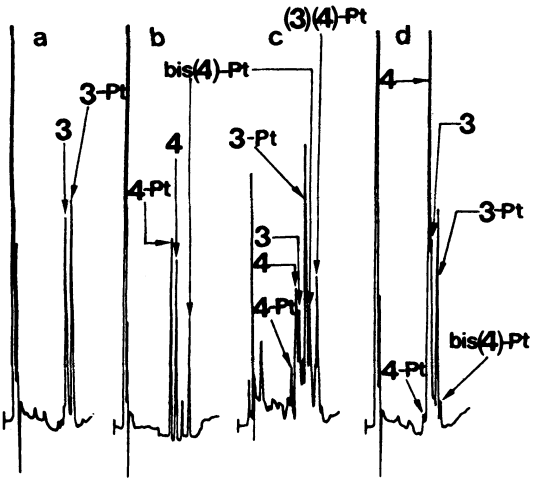

Fig. 2. Reverse phase HPLC analysis of the platination products from the 1:1 reaction of cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ 10^{-5}M with : a) **3** in $NaNO_3$ 0.5M ; b) **4** in $NaNO_3$ 0.5M ; c) **3** + **4** (1:1) in H_2O ; d) **5** in $NaNO_3$ 0.5M. Nova Pak, 5μ, C18 ; eluent A : triethylamine acetate 10^{-1}M, pH 6.3 ; eluent B : H_2O/CH_3CN 50:50 ; gradient 7% to 25% B in 3 min.

When the platination reaction is run with a stoichiometric mixture of both hexamers in water, one obtains the GG and AG chelates together with bis(**4**) and (**4**)(**3**) adducts (Fig. 2c). When the same reaction is run with the duplex **5** in $NaNO_3$ 0.5M, the GG chelate is highly predominant with a ratio [TG*G*CTA] / [TA*G*CCA] = 9 (the asterisks represent the platinum binding sites) (Fig. 2d). These results show that the selectivity of platination of GG vs. AG is similar for the single strands **3** and **4** (4$\pm$0.5) and the dinucleotides GpG and ApG (5$\pm$1, from $k_{1(tot)}$GpG and k_1ApG, Table 1). But it is larger for the duplex **5**, with a factor of about 9 for the GG vs. AG competition. One must note that these competitions involve two Gs for the GG sequence vs. one G for the AG sequence. We do not know yet the

ratio of the k_1 rate constants for the platination of a 3'-G and a 5'G within an oligonucleotide. However it is clear that the first platination of a G of a GG sequence is favored by a duplex structure.

Decanucleotides

We have compared the platination reactions of the two complementary decamers d(GCCGGATCGC) **6** and d(GCGATCCGGC) **7** with that of their duplex **8**.

The stoichiometric reactions of cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ **1** (10^{-5}M) with **6** in H_2O or in $NaNO_3$ 0.5M, gave similar mixtures of adducts (Fig. 3a). The main HPLC peak is that of the previously reported GG-chelate d(GCCG*G*ATCGC) **9** (26) but it only accounts for 30% of all the products formed. Six other complexes, each accounting for 5 to 10% of the products, could be separated by HPLC (Fig. 3b).

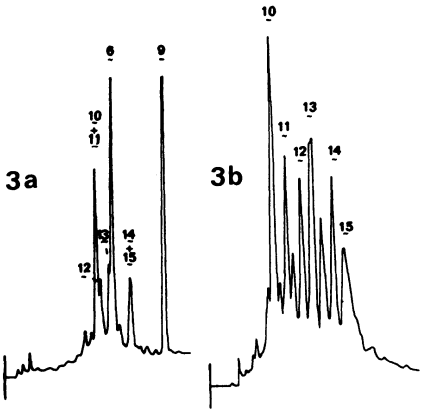

Fig. 3. Reverse phase HPLC analysis of the platination products of the 1:1 reaction of **1** (10^{-5}M) with **6** in H_2O : a) complete elution profile. Nucleosil 100Å, 5μ, C18, length 12.5cm, int. diameter 4.6mm ; eluent A : triethylamine acetate $2.5x10^{-2}$M, pH 6.5 ; eluent B : H_2O/CH_3CN 50:50 ; flow rate 1 ml.min^{-1} ; gradient 15% to 30% B in 15 min ; b) profile used for the preparative separation of the various GG-loops. Nucleosil 100Å, 5μ, C18, 12.5cm-7.5 mm; eluent A : triethylamine acetate 10^{-1}M, pH 6 ; eluent B : H_2O/CH_3CN 50:50 ; 2ml.min^{-1} ; gradient 17% to 25% B in 16 min. Complexes **10** and **13** were isolated as pure materials after a second HPLC separation of the components of the corresponding peaks.

Five contained one platinum atom and one contained two, as shown by atomic absorption spectroscopy. Upon digestion by deoxyribonuclease I and nuclease P1(5) followed by alkaline phosphatase, all of them gave cis-$[Pt(NH_3)_2(dG)_2]$, and the diplatinated one gave also cis-$[Pt(NH_3)_2(dGpG)]$ in accord with its formation that we found to occur at the expense of the GG-chelate **9**. All these adducts are G-G.cis-$Pt(NH_3)_2$ loops which could be identified thanks to a small endonuclease activity that we proved to be present in the commercial 3'-exonuclease snake venom phosphodiesterase (VPD, Worthington). This is illustrated with the case of the adduct **10**. After 2 min digestion with VPD, **10** releases only dpC, showing that platinum is bound to G(9) of **6** (27). After 40 min digestion, one dpT, one dpA, one dpG, one dpC and one dG equivalents are identified, leaving two dpG and two dpC equivalents undigested. It is known that endonuclease digestion of a phosphodiester group on the 5'-side of a platinated G is very much slowed down (4,5,27). Taking into account the sequence of **6**, the remaining platinated fragment must be cis-$[Pt(NH_3)_2(dpCpG^*)(dpCpG^*)]$. These results show that **10** is d(GCCG*GATCG*C). The same method allowed the identification of d(G*CCGGATCG*C) **11**, d(GCCGG*ATCG*C) **12**, d(G*CCGG*ATCGC) **13**, d(G*CCG*GATCGC) **15** (the loops having less than three nucleotides between the bound guanines are not digested and d(G*CCG*G*ATCG*C) **14**. The stoichiometric reaction of **1** (10^{-5}M) with **7** also gave a mixture of adducts (Fig. 4a,b) with two main components : d(G*CGATCCG*GC) **16** and d(GCGATCCG*G*C) **17**. Strikingly the proportions of **16** and **17** were reversed for the reactions in H_2O and $NaNO_3$ 0.5M, respectively 35%-15% and 15%-35% of the products formed (Fig. 4a,b).

The stoichiometric reaction (1:1) of **1** (10^{-5}M) with the duplex **8** gave comparable patterns in H_2O and $NaNO_3$ 0.5M (Fig.5), the latter conditions leading to a very small amount of G-G.cis-Pt loops (as expected from the UV melting profile of **8**, T_m=63°C). In these conditions, the two GG-chelates d(GCCG*G*ATCGC) **9** and d(GCGATCCG*G*C) **17** represent respectively about 50 and 25% of the reaction products. They show that 75% of the platinum is bound to the 50% of the guanines which belong to a GG sequence. This result seems

comparable to those obtained from DNA platinations (4,5).

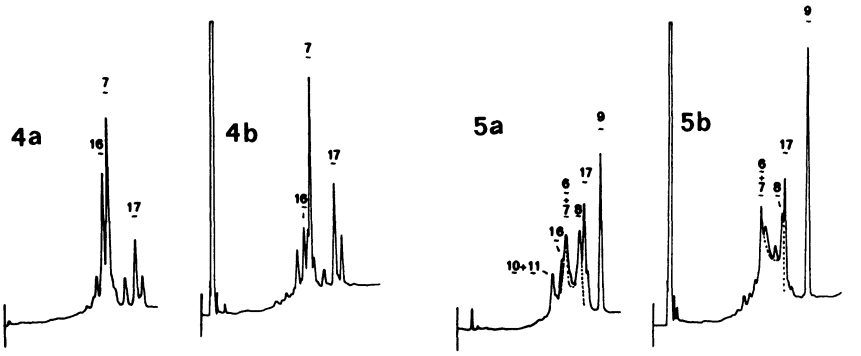

Fig. 4. Reverse phase HPLC analysis of the platination products of the 1:1 reaction of **1** (10^{-5}M) with **7** : a) in H_2O ; b) in $NaNO_3$ 0.5M. Same HPLC conditions as in Fig. 3a.

Fig. 5. Reverse phase HPLC analysis of the platination products of the 1:1 reaction of **1** (10^{-5}M) with the duplex **8** : a) in H_2O ; b) in $NaNO_3$ 0.5M. Same HPLC conditions as in Fig. 3a. In these elution conditions the duplex **8** is only partially dissociated and appears together with the single strands in the elution pattern represented by the dotted line.

It is noteworthy that the two GG sequences of **6** and **7** exhibit different behaviors for their platinations both in the single strands and in the duplex **8**. It is clear that the duplex structure favors the formation of the GG-chelates. However these results do not give direct informations about the rates of the first platination steps on the various Gs. Further work is in progress to try to correlate, at the single strand level, the various adducts formed with their monocoordinated precursors. One needs to compare the chelation aptitudes of the GG sequence when it is first platinated on the 3'or 5'-G.

DEFORMATION OF A DUPLEX STRUCTURE DUE TO A d(GpG). cis-$Pt(NH_3)_2$ CHELATE

For the last four years, two complementary approaches have been taken to address this point. The first was to investigate the formation of a duplex between a d(GpG)-platinated oligonucleotide and a complementary strand, and then to analyze its structure by spectros-

copic methods. The second was to use molecular mechanics calculations in order to find the most stable structure(s) compatible with the presence of the d(GpG)-adduct, whose metrical parameters became available from the X-Ray diffraction data of the complex cis-$[Pt(NH_3)_2\{d(pGpG)\}]$(28).

Three double-stranded platinated oligonucleotides have been reported and are indicated below with their melting temperatures compared to those of the unplatinated duplexes :

d(TCTCG*G*TCTC)-d(GAGACCGAGA) **18**,Tm=14°C(29°C) (29)
d(GATCCG*G*C)-d(GCCGGATCGC) **19**,Tm=28°C(55°C) (30)
d(GCCG*G*ATCGC)-d(GCGATCCGGC) **8**,Tm=49°C(58°C) (26)

The existence of a double-stranded structure was demonstrated by the UV and CD melting profiles and by the observation of the (GN1H, TN3H) imino proton resonances. The distortion of the platinated helix is reflected by the decrease in Tm, the smaller hyperchromicity effect observed upon melting, and the broadening of the signals of the imino protons which belong to the chelating guanines (26,29,30). ^{31}P (31,32) and CD (26,30,32) data suggest a great resemblance between the distortions of various platinated DNAs (at low r_b) and those of the platinated decanucleotide models, and support an overall B-DNA type structure for the latter. The 2D ^1H NMR studies of **18** (32) and **19** (33) revealed the presence of the characteristic features of the N7,N7, head-to-head, anti-anti d(GpG).cis-Pt chelate, particularly the typical N (C3'-endo) and S conformations of the 5'and 3'-guanosines, already encountered for the cis-$[Pt(NH_3)_2(dGpG)]^{2+}$complex (14,16). A careful NMR analysis done in the group of Prof. Reedijk, of the chemical shifts and coupling constants of **18**, suggested that the small distortion of the duplex could be described as a kink in the helical axis of about 40-70° (32).

Molecular mechanics calculations, done in the group of Prof. Lippard, have been performed on two platinated double-stranded oligonucleotides : [d(GGCCG*G*CC)-d(GGCCGGCC)]**20** and the duplex **18**. Two types of models were derived. The first one presents a mostly unperturbed helix axis but a large tilt of the 5'-end-coordinated guanine, with disruption of its GC pairing (model A)(34). The second one has a kinked helix axis, and in the "low-salt form" the base

pairing is intact on both sides of the kink (model C) (35,36). Both types of models exhibit the characteristic structure of the d(GpG).cis-Pt moiety (vide supra) and are stabilized by a hydrogen bond between an ammine ligand and the phosphodiester group in the 5'-position of the d(GpG) chelate. A distinct feature of the kinked model is a sugar N conformation of the cytidine on the 5'-side of the chelate (35,36). As all the duplexes studied contained a cytidine in this position, we decided to compare the NMR characteristics of duplex **8** with models calculated by molecular mechanics using the program AMBER (25,34,36).

The NMR analysis of **8** could be done, using multiquanta filtered COSY and NOESY techniques, according to the method of sequential resonance assignments which applies to right-handed, type-B, double-stranded oligonucleotides (37,38). The NMR data point to a remarkable similarity between **8** and **18**. Focusing on cytidine C(3) we have found that : i) the H1' couplings ($J_{1'2'} \simeq 4.5$Hz, $J_{1'2''} \simeq 6.5$Hz) show the existence of an equilibrium between N and S conformations for the deoxyribose, with a small predominance of the former (39) ; ii) the H2' signal is strongly upfield shifted suggesting the influence of the ring current effect of the adjacent guanine G(4) ; iii) the observation of a direct NOE between G(4)H8 and C(3)H2' reveals a proximity between these protons. Molecular mechanics calculations performed on **8** in the "gas phase", yielded models very similar to models A and C previously described for the duplex **18** (vide supra). However, calculations made for **8** in water solution showed that the aquation stabilizes structures lacking the direct hydrogen bond (NH---OP) between an ammine ligand and the 5'-phosphodiester group p(4) of the p(4)G*(4)p(5)G*(5) moiety. Several kinked and unkinked models were refined with different NH---OP distances and their structural characteristics were compared with the NMR data. The results are as follows : i) In all the unkinked models the C(3)H2' proton lies outside the shielding cone of the G(4) ring, and the C(3)H2'- G(4)H8 distances exceed 5.28 Å, data which cannot account for the H2' shielding and the H2'-H8 NOE observed ; ii) Three possible kinked structures resulted from the modelling : one with a direct NH---OP hydrogen bond and an N sugar pucker at C(3), and two

with a water molecule bridging one ammine ligand with the p(4) and p(5) phosphodiester groups. The latter two differ in their sugar pucker at C(3) being N or S respectively. In the two N models the C(3)H2' proton can experience the shielding of the G(4) ring (40,41) and the short C(3)H2'-G(4)H8 distance (1.99-2.15Å) (Fig.6a) should give rise to a very strong NOE, actually larger than that observed. The latter is smaller than the NOE between G(4)H8 and G(4)H3' separated by 1.78-2.08 Å. In the model with an S sugar pucker (Fig. 6b), the C(3)H2' lies farther from the G(4) ring and from the G(4)H8 (G(4)H8 C(3)H2' = 3.57 Å).

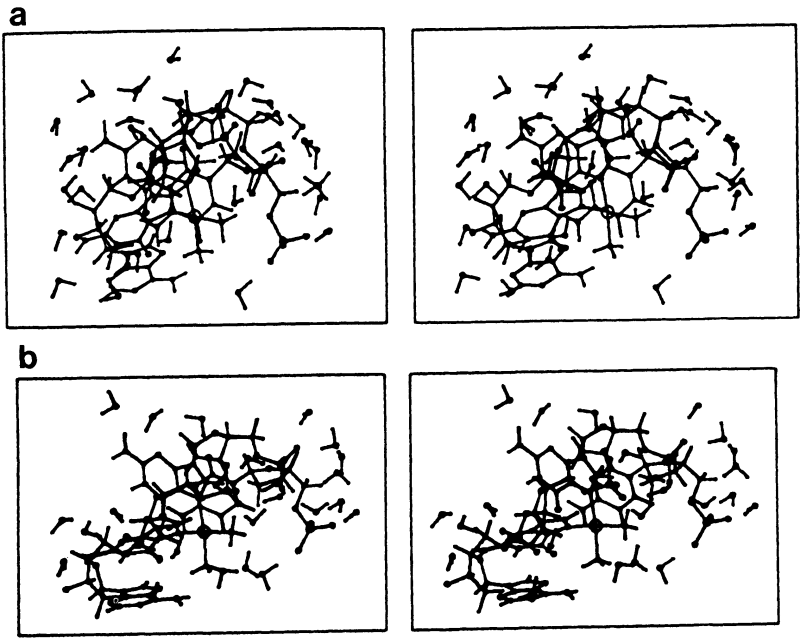

Fig. 6. Stereoviews of the C(3)p(4)G*(4)p(5)G*(5)p(6)A(6) fragment of two kinked models of duplex **8** in water : a) with direct ammine-phosphate p(4) hydrogen bond and C(3) in N conformation b) with NH---OP(p(4))=4.17Å and a water molecule bridging one HN_3 and the p(4) and p(5) phosphates C(3) in S conformation.

A mixture of three kinked structures could account for the N⇌S equilibrium indicated for C(3) as well as for the intermediate NOE observed between G(4)H8 and C(3)H2' .

CONCLUSION

Oligonucleotide model studies suggest that the selectivity of the first platinum binding to a G within a $(dG)_n$ sequence is favored i) by the presence of two aqua ligands on the metal, ii) by steric factors which are effective in a double-stranded structure. More work is needed to get a better understanding of the contributions of these various factors.

A model study with the platinated duplex [d(GCCG*G*ATCGC)-d(GCGATCCGGC)] based on a ^{1}H NMR analysis and molecular mechanics calculations in water, shows that the structure is kinked.

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MACROMOLECULAR ANTITUMOR DRUGS: PHARMACOLOGY AND BIOLOGY

J. Drobnik

After 20 years of research we got insight into the molecular pharmacodynamic of platinum drugs, i.e. we can draw the structure of the complex formed between the platinum atom and the DNA molecule which is responsible for the cytostatic effect. We have also collected enough evidence supporting the hypothesis that the block of cytokinesis is part of a complex cellular response to specific DNA damage induced by platinum coordination compounds with cis-position of leaving ligands.

However, the mystery of "complex at target" remained unsolved. We have no evidence about the nature of the final form of the platinum complex which after its quite long way from the site of application reaches the DNA in the nucleus of the cancer cell. Many years ago we suggested that due to the low chlorine concentration inside the cell platinum (II) may be there in the form of an aquo- or hydroxo complex. Today we do not see any reason to change this suggestion even when facing the broad selection of so called second generation drugs.

One may argue that this may be true for both leaving ligands but not for aminoligands which are more stable and can therefore go with the platinum atom all its way to the target being responsible for the different effect of individual members of the drug family.

This may or may not be true. Brian Robins, working at the Chester Beatty Institute for Cancer Research with ^{14}C ethylenediamine as the aminoligand and with ^{191}Pt in dichloroethylenediamine platinum(II) complex, showed clearly that both nuclides became separated in vivo, indicating the detachment of the amino ligand from the platinum atom. We suggested an explanation by the sulfur trans-effect which may occur when the drug is inactivated by irreversible binding to sulfur

containing molecules, e.g., to methionine. If so, the detachment of aminoligand may not be relevant to the therapeutic activity and the platinum which binds to the DNA may be accompanied by the original aminoligand.

However, even in such case we suggest that aminoligands of antitumor platinum complexes are irrelevant to the molecular process at the DNA level. Nevertheless, there is quite a difference in the biological activity of various platinum drugs related to their different chemical structure. We suppose that this is because the structure solely modulates the molecular pharmacokinetics affecting the fate of the drug in the body.

The whole second generation of platinum drugs may be used as an example. Its selection was pointed against the nephrotoxicity - as it was believed one time that it was the nephrotoxicity which sets limits to the use of cisplatin. As a result the "reactivity window" was extended to both sides introducing "fast drugs" quickly inactivated and "slow drugs" which pass unchanged through the kidney as cisplatin does but differing from it in their inability to react with kidney structures during the passage.

Summarizing our hypothesis, we consider different ligands utilized in the construction of platinum antitumor drugs mainly as a tool of controlling the fate in the body but not the mechanism of the specific interaction with DNA.

Starting from this point, we were looking for an even more powerful means of regulating the pharmacokinetics. The macromolecular form was our choice for the following reasons: The body regulates life processes - including malignant growth and its control - mostly through macromolecules, whereas our drugs are small molecules. The activity of a macromolecule can be easily controlled in space and time: in space due to its limited passage through compartmental barriers and in time by its degradation. This is the way how the nature works and we have to learn from it.

However, inspecting the Pharmacopeias and catalogs of pharmaceutical companies, we find practically no macromolecular drugs used in clinics despite the flood of papers and patents. We see two reasons:
- there are many variables characterizing a synthetic polymer which

makes the choice and characterization very difficult
- our knowledge of the fate of synthetic polymers in the body is far from being sufficient.

These two groups of problems are in connection, since the fate of the polymer in vivo is a function of its properties. On the other hand, exact answers in both directions are strongly needed by authorities registering drugs for clinical use.

Looking for the carrier polymer, we have to decide if the polymeric drug made from it is used for research and solution of theoretical questions of pharmacokinetics, or if it is designed for the application to patients. In the former case a polymer resistant to biodegradation will provide better results. However, safety of the patients imposes a strong requirement on total digestion of the polymer, i.e. its degradation to products which are safely metabolized or excreted.

There are several false beliefs in this regard. We may find statements that molecules - polymers or their degradation products - of a size which falls below the filtration limit of glomerules (approximately corresponding to a mol. weight from 15 to 25 thousands) are safely and completely eliminated from the body. We have shown that this is not the case for two reasons:

First, polymers are subjected to endocytosis by various cells. They accumulate in lysosomes and if they are resistant to degradation by the complex of lysosomal enzymes they stay there. The diffusion limit of the lysosomal membrane is as low as a mol. weight about 200.

Second, successful filtration across the glomerular membrane does not ensure safe elimination. We demonstrated an important tubular resorption of polymers from the primary urine which is controlled by the chemical structure of side chains of the polymer. This means that a polymer which does not accumulate in the kidney may do so if a certain chemical group is attached to it.

Third, polymers or their degradation products which are captured in macrophages and are not completely digested in their lysosomes may be presented as antigens causing allergy by repeated administration.

For these reasons we used two polymers as carriers of the platinum complex: PHEA - α,β-poly[N(2-hydroxyalkyl)-D,L-asparagine], which is resistant to biodegradation, and PHEG - α-poly[N(2-hydroxy-

alkyl)-L-glutamine], which is hydrolyzed by several enzymes.

Having decided about the type of the polymer, we come to the question of bonding the active group to the polymeric backbone. In general, we suppose polymers to stay outside the cytoplasma since till now nobody has been able to prove the opposite. Therefore, all drugs whose effect is operative inside the cytoplasma or caryoplasma have to be released from the carrier polymer.

This question is not as clear-cut with platinum drugs for reasons mentioned above, i.e., because we do not know the chemical nature of the complex which attacks DNA within the cell nucleus. If the exchange of aminoligands shown by Robins results in an active species reacting with DNA in a proper way, then the platinum atom can be attached to the polymer even through nitrogen as it is the case in polymers prepared by Carraher. In fact, studying the pharmacokinetics and metabolism of such polymers, information can be obtained contributing to our understanding of the above mentioned mystery of the "complex at target".

Since this has not been done yet, we felt safer using a well-known second generation complex TMA [trans-1,2-diaminocyclohexane trimellitato platinum(II)] attached to the polymer by an easily hydrolyzable ester bond. Lacking any idea about the optimal rate of release, we prepared two types of this bond.

A successful design and synthesis of the polymeric form is by no means a guaranty that the drug will go. Even good substance can be killed by improper testing.

The routine testing by intraperitoneal application cannot be used with polymers. Their crossing of compartmental barriers is very slow and therefore there is a big difference between their effects after i.p., i.v., i.m., s.c., and other routes of application. This is the advantage of the polymer that it stays for a long time in the compartment of application. However, by routine testing of acute toxicity and by calculating DL_{50} the macromolecular form can be rejected, since the LD_{50} value could be lower than with a free drug because of longer residential time in the compartment.

Shortly, the specifity of the polymeric drug fate in the body - and this is why we are developing them - must be carefully considered

by the development of specific testing methods.

At the end, please allow me to make a few comments concerning the so-called targeting. A different affinity to various parts of the body is an inherent property of any polymer. However, it should be kept in mind that an attachment of a biologically active or signal group to it may substantially change this affinity and consequently the biodistribution of such a modified or "functionalized" polymer.

The addition of specific "homing" groups to macromolecular drugs has been very popular in these years. Considering immunity, we are not enthusiastic about the antibodies, Fab fragments, lectins and other proteins as targeting groups for therapeutics which have to be given several times to the patient. Sugars, hormones and other small molecules specific for certain cell surface receptors seem more promising.

It was possible in this contribution to summarize just the most important features of polymeric drugs. Nevertheless, even this is enough to demonstrate the necessity of an entirely new approach to their design, synthesis, identification and testing. The same holds for clinical use. One may venture a prediction that the best effect could be expected in local application where an elevated level of the active component in one compartment is beneficial, or in cases where a slow release for a long period of time is wanted. However, it is the task of clinicians to look for the optimal application of macromolecular drugs.

UNUSUAL HAIRPIN-LIKE CONFORMATIONS OF OLIGONUCLEOTIDES INDUCED BY PLATINUM ANTI-CANCER DRUGS

L.G. Marzilli, C. Spellmeyer Fouts, T. Page Kline and G. Zon

The great efficacy of Pt-anti-cancer drugs[1,2] combined with strong The great efficacy of Pt-anti-cancer drugs[1,2] combined with strong evidence that these drugs target DNA[3] has prompted numerous studies of the binding of Pt complexes to DNA.[2,4-7] The principal type of lesion is known to be Pt intrastrand crosslinked GpG moieties.[2,4-7] Our finding that active (e.g. cis$Pt(NH_3)_2Cl_2$) but not inactive (e.g. trans$Pt(NH_3)_2Cl_2$) compounds induced an unusual downfield ^{31}P NMR signal in DNA[8,9] was followed by similar findings for nucleosomes,[9] polynucleotides,[9,10] and oligonucleotides,[11-17] but only for those species with adjacent 6-oxopurine bases. There are well-defined relationships between structural changes and ^{31}P NMR chemical shifts.[18] A knowledge of structure is essential for understanding the reason for the potency of this class of drugs.[2-16] Indeed, considerable effort has been expended to obtain relevant crystals and only recently has there been some success but with cis$Pt(NH_3)_2$ adducts of di- and tri-nucleotides.[19]

As part of a program aimed at understanding the relationship between ^{31}P NMR spectra and the consequence of DNA platination, we had treated the self-complementary tetradecadeoxyribonucleotide, $d(TATGGGTACCCATA)_2$ ($5' \rightarrow 3'$) [1], with several Pt drugs at 12 °C and 0.1M $NaNO_3$, conditions stabilizing the duplex form. Although the ^{31}P NMR results we obtained with Pt adducts of 1 were novel, more importantly several types of evidence reveal that the products must have a previously unrecognized hairpin-like conformation.

The primary evidence for this conclusion rests in the ^{31}P NMR spectra of the adducts as illustrated in Figure 1 for the Pt(en) adduct, 1-Pt. All thirteen possible forms of 1-Pt labeled with ^{17}O at a different phosphate group were synthesized.[11] Since ^{17}O labeling reduces the intensity of the

^{31}P NMR signal,[11,20,21] the ^{31}P NMR signals can be assigned, Figure 1. For all known cases, the ^{31}P signal for Pt crosslinked NGpGN adducts occurs at ca. -3.0 ppm (relative to TMP)[4,9,12-17] and is fairly insensitive to temperature, pH, duplex formation, etc. (N = nucleotide, TMP = trimethyl phosphate).[12,22] Crosslinking between the two 3' G residues in 1-Pt should lead to a downfield position for P5 as observed, Figure 1. Likewise, treatment of d(TGGGTACCCA)$_2$ [2] with Pt(en)Cl$_2$ leads to the Pt adduct, 2-Pt, with the signal corresponding to P5 at -2.92 ppm. For 2-Pt, there is a clear NOE between ^1H NMR signals at 8.87 and 8.70 ppm, a finding characteristic of Pt crosslinked GpG moieties.[4,12,14,22] For 1-Pt, the observation of only the required number of ^{31}P signals (13), the simplicity of the ^1H NMR spectrum in the aromatic region and the electrophoretic analysis (see below) all point to one major product for 1-Pt. There is no significant amount of an adduct with a crosslink between G4 and G5.

A striking feature of the ^{31}P spectrum of 1-Pt is the signal at -2.57 ppm which is unambiguously assigned to P8, in a phosphate group well removed from the Pt lesion. Likewise, the signal corresponding to P8 in 2-Pt occurs at -2.44 ppm. The downfield position of this resonance is unprecedented for drug adducts of DNA species unless the phosphate group is at the binding site.[23-25] Most such downfield shifted signals can be explained by a combination of structural changes in the sugar-phosphate backbone and the anisotropic deshielding effect of aromatic drugs intercalated into DNA.[23-25] The -2.57 ppm position of ApC is thus anomalously downfield and suggests a major distortion of the backbone.

The CD spectrum of 1-Pt is greatly reduced in intensity compared to that of 1; i.e. $[\Theta]_{280}$ is 4.0 vs. 6.9 (x 10^3 deg $M^{-1}cm^{-1}$). In contrast, when duplex integrity is maintained on platination by anti-cancer drugs, $[\Theta]_{280}$ actually increases, e.g., duplex 3-Pt (single-strand d(TCTCGGTCTC)cisPt(NH$_3$)$_2$ [3-Pt] and its complement).[22] Several other lines of evidence that 1-Pt is not a duplex follow. First, the UV melting profile of 1-Pt is featureless, with absorbance rising slowly with temperature. In contrast, 1 exhibits a broad transition in 0.1M $NaNO_3$ and the characteristic duplex $\rightarrow$ hairpin profile[26-32] in low salt. Second, 1-Pt has a relative mobility (RM) of 1.18 versus that of duplex 3 $\equiv$ 1.00. The duplex form of 1 has RM = 0.89 whereas, as expected,[33,34] the hairpin form has RM =1.20, similar to that of 1-Pt. In contrast, duplex 3-Pt has RM =1.02. Third, 1-Pt has

sharp imino ^1H signals at 12 to 14 ppm consistent with either a duplex or hairpin-like form.[35,36] However, it also has D_2O exchangeable protons giving broad signals at ca. 11.1 and 12.0 ppm. Such upfield signals are observed in hairpins and are assigned to imino H's not involved in Watson-Crick base pairing but which are protected from rapid exchange with H_2O.[33,36-38] The imino signals of 1-Pt are observed even at low concentrations, an observation characteristic of hairpins.[33,36-38]

Although the similarities of the properties of 1-Pt with those of hairpins are compelling, we feel the term "hairpin-like" is a more appropriate description for the conformation of 1-Pt for several reasons. First, 2-Pt has a nearly identical ^{31}P spectrum except for the absent phosphate groups. It lacks much of the stem region of 1-Pt and has imino signals only below 25°C. Imino signals are observed for 1-Pt up to 65°C. Thus, the stem of 1-Pt stabilizes the structure but is not necessary for the loop. Second, single-stranded 3-Pt has RM = 0.84 vs. 1.10 for single stranded 3. 3-Pt also lacks imino signals, which is understandable since it is not self-complementary. However, our ^{31}P NMR studies on 3-Pt reveal that, in addition to the downfield signal at -2.60 ppm assigned to the crosslinked GpG moiety,[16] the unassigned signal at -3.10 ppm is from CpTCGG, a group well removed from the lesion. As for the ApC signal at -2.57 ppm for 1-Pt, this downfield CpT signal is temperature sensitive and moves upfield towards the normal shift range (ca. -4.2 ppm) for an undistorted phosphodiester group in DNA. This finding suggests that both in 1-Pt and in 3-Pt, the Pt moiety can induce distortions which exist at physiological temperatures but which are eliminated at elevated temperatures. However, it is noteworthy that the downfield GpG signal which arises from the Pt crosslinking is not sensitive to temperature and remains downfield in both 1-Pt and 3-Pt at elevated temperatures. Thus the two types of downfield signals are readily differentiated. Since 3-Pt is not self-complementary, the distortions induced by the Pt do not require the existence of a stem. Third, hairpins have conservative B-DNA type CD spectra[32] and exhibit appreciable UV absorbance increases with increasing temperature. As stated above, 1-Pt does not have these characteristics. Finally, hairpins do not have ^{31}P signals significantly shifted from the shift range for DNA (ca. -4.0 to -4.5 ppm).[39]

These findings lead us to believe that an important feature of the distortion is the interaction of the flanking sequences with the positively charged Pt-amine moiety. The recent X-ray studies[19] reveal H-bonding interactions between phosphate groups and the amine. The charge and H-bonding may distort the single-stranded structures but in turn will stabilize the single-strand relative to the duplex form. Such single-strand stabilization can be used to rationalize the apparently conflicting observations of a relatively undistorted structure for duplex 3-Pt combined with a duplex → coil transition at a lower temperature than for duplex 3.[22] We find other Pt drugs give adducts with 1 similar to 1-Pt. It is interesting to speculate that palindromic DNA regions involved in gene control[40] may be destabilized by the Pt drugs. However, much more study is required to unravel fully the molecular and biochemical mode of action of these important anti-cancer agents.

ACKNOWLEDGMENT

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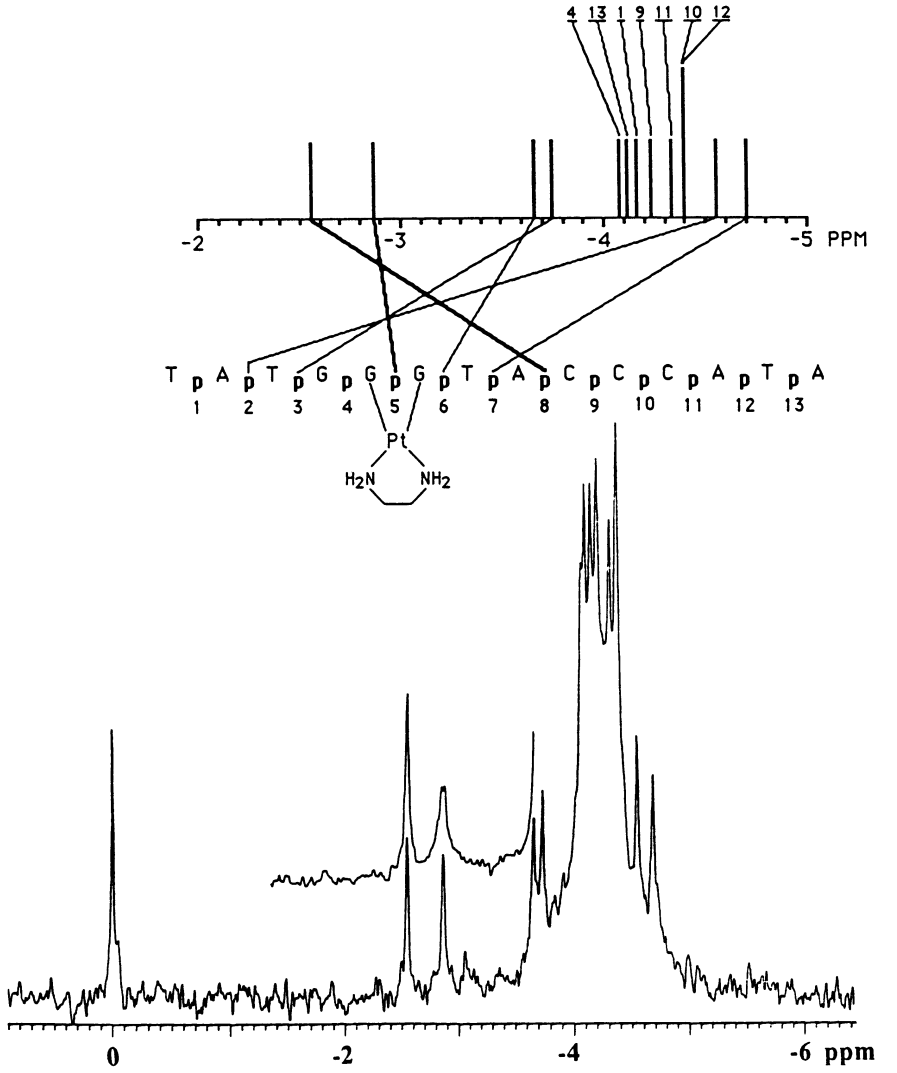

Figure 1. Bottom: The 81.01 MHz $^{31}P\{^{1}H\}$ NMR spectrum of unlabeled 1 -Pt (0.010 M PIPES, 0.100 M $NaNO_3$, 0.001 M EDTA , pH 7.0, 25°C). Inset shows the downfield region for 1 - Pt labeled with ^{17}O at P5, one of 13 such experiments. Top: Schematic summary of the assignments of all the ^{31}P signals by ^{17}O-labeling studies.

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CISPLATIN-RADIATION INTERACTIONS IN TUMOURS AND NORMAL TISSUES

A.C. Begg, F.A. Stewart, L. Dewit and H. Bartelink

Introduction

Studies were undertaken to define the relative importance of mechanisms by which cisplatin can enhance the effects of radiation treatments. In a mouse tumour and in three different normal tissues, the magnitudes of (a) cell killing by the drug, (b) radiosensitization, (c) inhibition of X ray damage repair, and (d) inhibition of proliferation were investigated. The last two mechanisms would be particularly important in fractionated treatments. These studies were therefore designed to test whether a therapeutic advantage could be obtained by combining cisplatin and irradiation and, if so, by which mechanisms this was achieved.

Materials and Methods

The tumour used in these studies was the RIF1 sarcoma grown as either monolayer log phase cultures or subcutaneous tumours in mice (1). Cultures were given 1 hr cisplatin exposures at different times before irradiation and the fraction of surviving cells assessed by colony formation. Sublethal damage repair (SLDR) was measured by the increase in survival when a dose of 5 Gy was split into 2 doses of 2.5 Gy separated by 1 to 3 hr. Potentially lethal damage repair (PLDR) was assessed by the increase in survival occurring after a single dose of 5 Gy when plating was delayed by 3 hr. In vivo, C3H/Km mice bearing subcutaneous RIF1 tumours on the dorsum were given intraperitoneal injections of cisplatin 0,5 hr before local irradiation of 6 mm diameter tumours. Irradiations were given under ambient conditions or with tumours clamped 5 min. before and during irradiation to render all cells hypoxic. Damage was assessed by tumour growth delay to 2mm above the diameter at treatment.

Kidney damage was assessed by clearance of ^{51}Cr radioactivity from plasma of mice given i.p. injections of ^{51}Cr-EDTA. The damage was assessed approximately 30 weeks after treatment. Damage to the duodenum was assessed by the crypt microcolony assay of Withers and Elkind (2) after partial abdominal irradiation. Rectal damage in mice was assessed by the incidence of stenosis 40 weeks after local irradiation of the colorectum. All in vivo irradiations (tumour, kidney, duodenum, rectum) were carried out on unanaesthetized mice.

Results

Tumour. A 1 hr exposure of only 1 μg/ml (3 μM) cisplatin to log phase RIF1 cells reduced the surviving fraction to 10%, indicating that this is a drug sensitive cell line. Cells irradiated at the end of a 1 hr exposure were also more sensitive to irradiation than untreated cells. Enhancement ratios increased with cisplatin dose up to a value of 2.2 after 1.2 μg/ml (3). SLDR inhibition by cisplatin was also observed (Fig. 1). A 1 hr drug exposure given immediately before the first of 2 X ray doses completely inhibited SLDR, whereas drug given 24 hr earlier had little effect. In

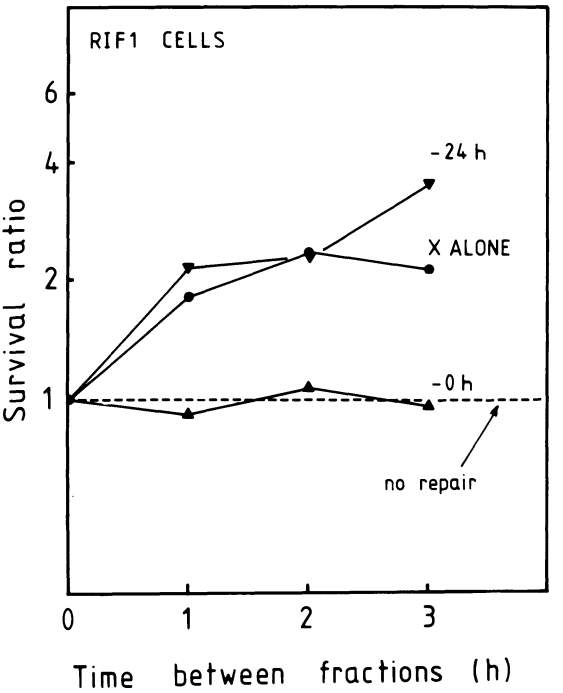

Figure 1. One hour cisplatin treatments inhibit SLDR if given immediately before but not 24 h before 2 X ray doses.

contrast, parallel studies showed that PLDR was increased by cisplatin treatment of log phase cells, with the net effect (SLDR + PLDR) being a small but insignificant repair inhibition over 3 hr. These results suggest that repair inhibition is not a major factor in enhancement of radiation effects by cisplatinum in these cells. Similarly, studies on the proliferation rate of cells surviving cisplatin treatment showed little growth rate perturbations, indicating that inhibition of proliferation is also not an important factor in combined cisplatin-radiation treatments.

Subcutaneous RIF1 tumours were radiosensitized by a single i.p. cisplatin injection of 6 mg/kg given 0,5 h before local irradiation of clamped (hypoxic) tumours (Fig. 2). Radiosensitization was also seen under ambient conditions. Comparison of data for 1 and 4 X ray fractions (Fig. 2) indicated little inhibition of repair of radiation damage between fractions, despite marked radiosensitization with both schedules. Repair inhibition was observed, however, when 2 mg/kg cisplatin was given 0.5 h before each of the 4 X ray fractions, although, in general, radiosensitization and independent cell killing appeared to be the two largest contributors to the observed enhancements in these tumours.

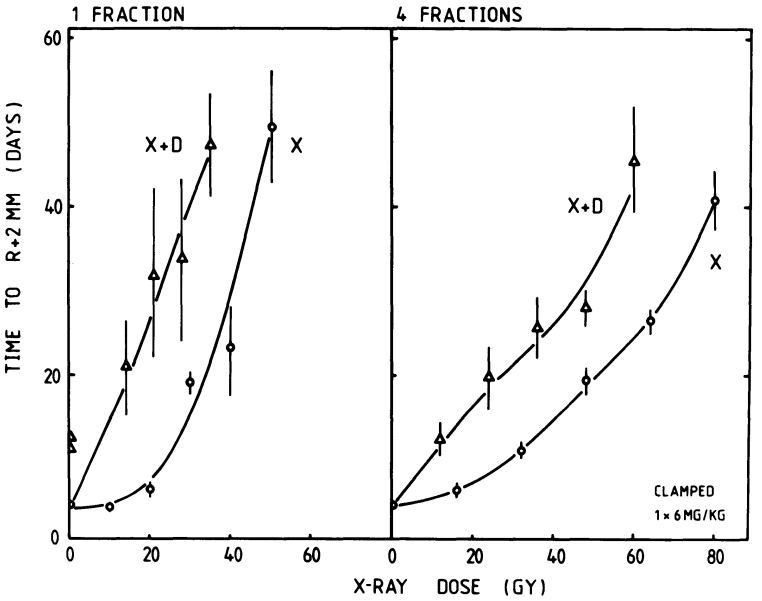

Figure 2. 6 mg/kg cisplatin sensitized RIF1 tumours in vivo to 1 or 4 fractions X rays. Enhancement ratios ranged from 1.4 - 3.1.

Kidney. Radiation damage in the kidney appeared late (after 5 months) and increased progressively at a rate dependent on the dose given (4). Cisplatin damage, in contrast, was maximum within a few days of treatment and then diminishes over the following few weeks to stabilize at a dose dependent level. Some damage persisted for many months after doses in excess of 4 mg/kg (4). Treatment with 2 x 4 mg/kg cisplatin increased radiation damage by a factor 1.3, independent of the X ray fractionation schedule (Fig. 3). This constant enhancement ratio argues against inhibition of repair of radiation damage by cisplatin, which would be expected to result in greater enhancements with greater number

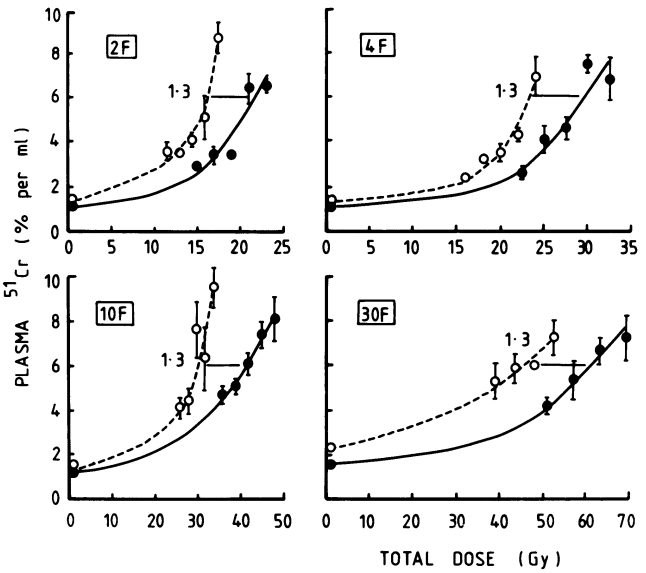

Figure 3. Dose response curves for renal damage 30 weeks after 2-30 fractions X rays alone (o) or combined with 2 doses of 4 mg/kg cisplatin (o). Enhancement ratios were 1.3 for all schedules.

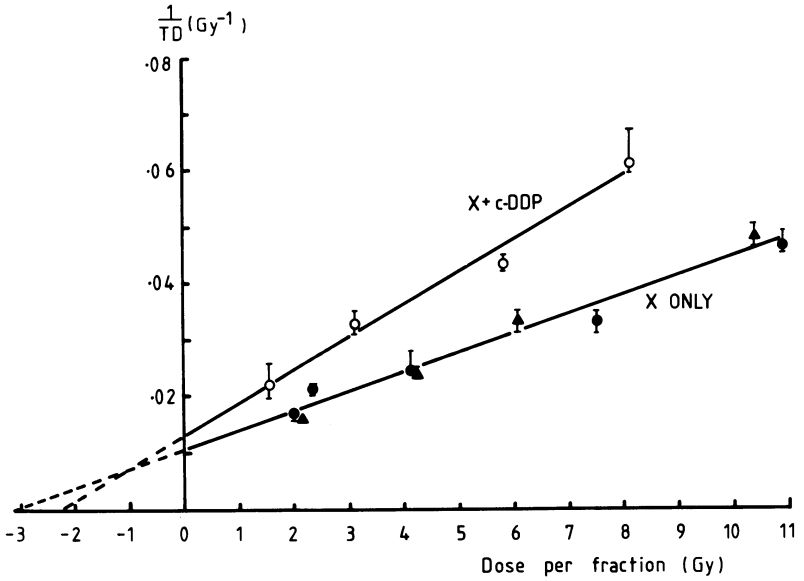

Figure 4. Reciprocal total dose plot shows little change in the α/β ratio (x intercept) for combined treatments, implying no modification of the X ray dose response curve by cisplatin.

of fractions, i.e. repair opportunities. This is confirmed by constructing reciprocal total dose plots (Fig. 4) in which the x intercept gives the value of - α/β, a measure of underlying survival curve shape for the target cells. These data therefore suggest that independent killing by the two agents occurred with little modification of the X ray dose response curve. Proliferation inhibition by the drug is expected to be unimportant in such a slowly proliferating organ.

In further studies, it was of interest that mice with pre-irradiated kidneys treated 3 to 6 months later with cisplatin showed a considerably enhanced sensitivity to the drug compared with mice treated within 1 month of irradiation (5). This serves as a warning against retreatment with cisplatin of patients given even low doses of radiation to the kidney many months previously.

Intestine. A single dose of 8 mg/kg cisplatin i.p. given 0,5 h before the first irradiation increased duodenal crypt cell killing (Fig. 5). For the 15 fraction X ray schedule (interfraction

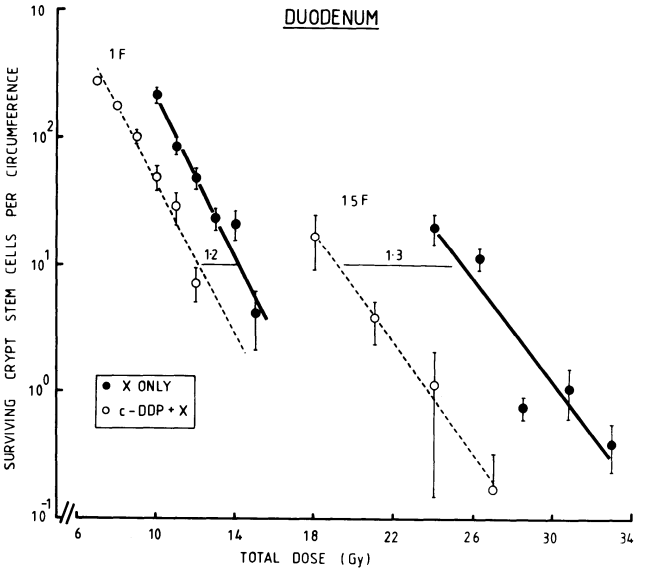

Figure 5. 6 mg/kg cisplatin increased the response of duodenal crypt cells to 1 or 15 fractions X rays. Modelling studies indicated primarily additive killing plus a small component of repair inhibition.

interval 3 h), the enhancement ratio was greater than that for single dose irradiations. Modelling studies, however, indicated that this small increase in the enhancement ratio is consistent with independent killing by the two agents (6,7), although a small contribution from repair inhibition cannot be ruled out. Cisplatin

was also shown to partially inhibit proliferation of surviving crypt cells, although this effect was not large. The major contributor to enhanced combined treatment effects in the duodenum, therefore, as in the kidney, appears to be additive killing by drug and X rays.

In the rectum, little or no increase in damage (stenosis) was observed when a 6 mg/kg cisplatin treatment was added to single doses of irradiation, independent of time or sequence (8). In fractionated X ray treatments using the same cisplatin dose, (Fig. 6) there was a suggestion of a slight reduction in repair, although this was not significant (L.Dewit, Y.Oussoren, H.Bartelink, H.D.Thames, 1987, unpublished). The effect of cisplatin on proliferation in this organ has not been studied. The rectum, therefore, seems particularly resistant to cell killing or radiosensitization by cisplatin.

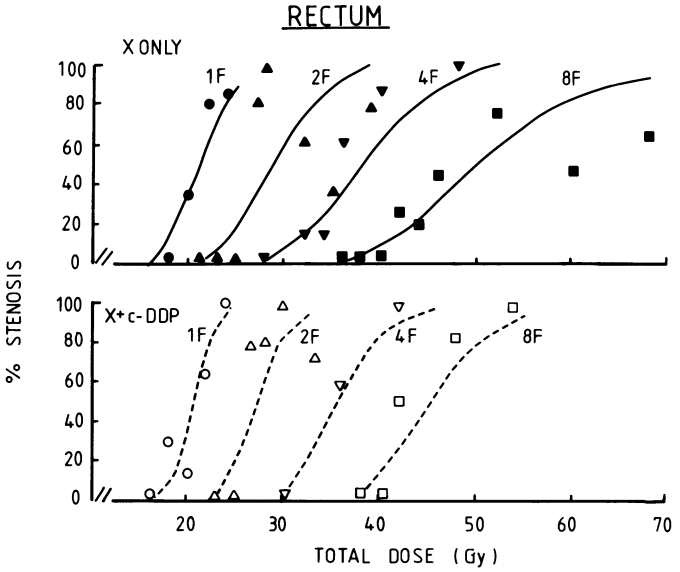

Figure 6. 6 mg/kg cisplatin had little effect on the response of mouse rectum to 1-8 fractions X rays.

Discussion

The data presented here show that the enhancement of radiation damage by cisplatin in the tumour (enhancement ratios 1.4-3.1) was greater than that in the three normal tissue studied (enhancement ratios 1.0-1.3). Table 1 summarizes the relative importance of the factors contributing to increased combined treatment effect. The two main factors responsible for the therapeutic gain were greater cell killing and greater radiosensitization in the tumour compared with those in normal tissues. The roles of repair inhibition and proliferation inhibition were smaller and of questionable importance. It should be pointed out

that the tumour studied here was sensitive to cisplatin, and therapeutic gains in less sensitive tumours may be smaller or absent. Present and future research is therefore being directed at finding simple and reliable methods of measuring or predicting the sensitivity of a tumour to killing and/or radiosensitization by the drug. One such method is the measurement of cisplatin-DNA adducts using specific antisera (see Terheggen et al., this conference).

Table 1

	RIF1 tumour	Kidney	Duodenum	Rectum
Killing	+++	++	+	-
Radiosensitization	++	±	±	-
Repair inhibition	+/-	-	±	±
Prolif. inhibition	-	-	±	-

-, no effect
±, very small effect
+/-, sometimes effect, dependent on conditions
+, ++, +++, significant effects

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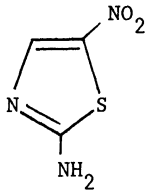
LINKAGE ISOMERS OF PLATINUM COMPLEXES OF ANT (2-amino-5-nitrothiazole) DIFFER IN THEIR DNA-BINDING AND RADIOSENSITIZATION

N.P. Farrell and K.A. Skov

As part of our studies on targetting radiosensitizers to DNA using platinum complexes, we have studied the properties of the ligand 2-amino-5-nitrothiazole (ANT):



The free ligand is a radiosensitizer (1) and presents possibilities of more than one binding site. Studies on its palladium complexes had shown a solvent-dependent linkage isomerism, ring bound trans-$[PdCl_2(ANT)_2]$ being formed in MeOH and a chelate formed from the ring nitrogen and exocyclic amine $[Pd(ANT)_2]Cl_2$ being observed in water (2). For platinum, linkage isomers of trans-$[PtCl_2(ANT)_2]$ (coordinating atom ring nitrogen, isomer R, or exocyclic amine, isomer A,) have also been isolated and linkage assignment made on the basis of IR. Studies in mammalian cells show that the linkage isomers differ in both their radiosensitizing properties and toxicity. The ring bound isomer binds better to DNA, (3) as judged by inhibition of restriction enzyme activity on plasmid DNA. (4) The higher toxicity of R (3) is presumed due to the DNA binding. Isomer R is also the most efficient radiosensitizer, supporting a correlation between DNA-binding and sensitizing activity for metal-sensitizer targetted compounds (5,6). Isomer R is a better sensitizer than ANT alone (enhancement ratio for 100 μM isomer R: 1.6; isomer A: 1.16; for ANT = 1.18) (3); it is

also better than that previously reported for trans-plant (7) which may have been a mixture of R and A. In vivo assessment of this DNA targetted radiosensitizer is planned.

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COMPETITION BETWEEN DNA AND ORTHOPHOSPHATE FOR COORDINATION WITH PLATINUM(II) COMPLEXES

E. Holler

INTRODUCTION

Orthophosphate is a major inorganic anion in blood and tissue. Its potential to coordinate to platinum complexes is of interest with regard to therapeutical effects.

MATERIALS AND METHODS

Salmon testis DNA was purchased from Sigma. It was dialysed against several changes of a solution of 10 mM KNO_3 before use and stored at -20° C. The racemate of 1,2-bis(4-fluorophenyl)ethylenediamineplatinum(II) (i) was a gift of Dr Schönenberger/Regensburg, cis-diamminedichloroplatinum(II) (ii) was a gift of Degussa/Frankfurt and all other chemicals were of highest available grade from Merck/Darmstadt. Compounds (i) was in its sulfate form and reacted as a diaqua complex (1). Cis-diamminediaquaplatinum(II) (iii) was prepared by the reaction of (ii) with $AgNO_3$ (1 : 2 mol : mol) over night at 37° C. All solutions used in the reactions contained 10 mM KNO_3 and had a pH 5.5 - 5.7. Platination of DNA was carried out in a 1 cm quartz cell directly in a suitable UV-photometer at 37° C. The reaction was followed at wavelength 280 nm exhibiting a hyperchromic effect (2).

RESULTS

Orthophosphate inhibited the reaction between salmon testis DNA and platinum(II) complexes (i) and (iii) as indicated by a decrease in the reaction amplitudes of both conformational rearrangements. These rearrangements give rise to a UV hyperchromicity (2). The dependence of the amplitudes is different for the two platinum complexes (Figure 1). In the case of (i), the fast rearrangement

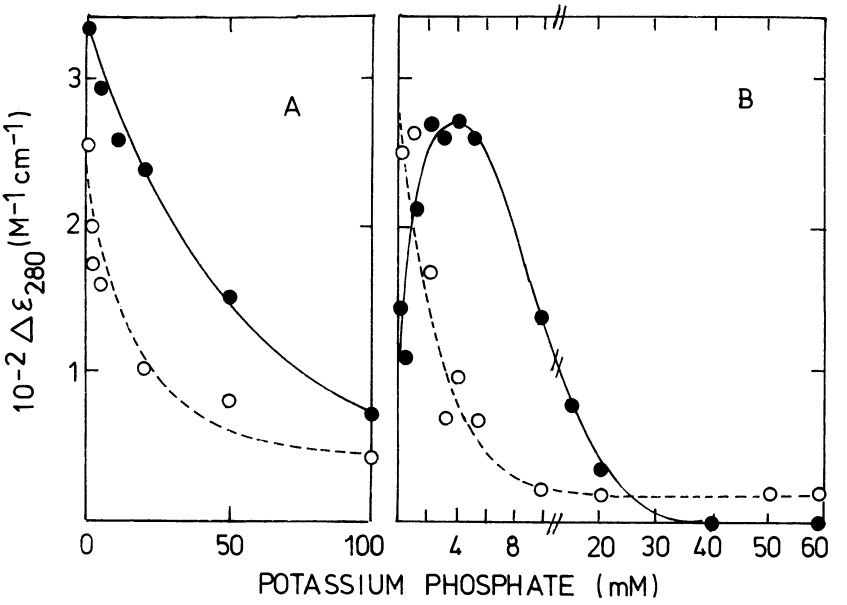

Fig. 1. Inhibition of DNA platination by orthophosphate. The difference of A_{280} was measured between 0.5 mM solution of salmon testis DNA (reference) and 0.5 mM solution of salmon testis DNA containing 0.05 mM platinum(II) complex (sample). Panel B, complex (i). Panel A, complex (iii). Sample and reference contained varying amounts of potassium phosphate. After addition of the platinum(II) complex, the difference in A_{280} was recorded as a function of time. The kinetics were biphasic. They were treated as a superposition of two exponential decays as described (1) and reaction parameters determined. The two reactions have been called the fast and slow "rearrangements" (1, 2). The reaction amplitudes are (-o-) for the fast and (-●-) for the slow rearrangement.

showed a decrease of the reaction amplitude that followed phosphate concentration in a hyperbolic fashion with a half-maximum value of 2 mM phosphate (Panel B). The dependence for the slow rearrangement was biphasic with half maxima at 1 - 2 mM and 10 mM phosphate for the ascending and descending branches, respectively. In the case of (iii), the amplitudes for both the fast and the slow rearrangements followed hyperbolic dependences with half maxima at 10 mM and 50 mM phosphate, respectively (Panel A).

In the absence of DNA, the reaction between phosphate and either (i) or (iii) gave rise to difference spectra (platinum(II) complex, reference/platinum(II) complex and phosphate, sample). They had maxima at 261 nm and 268 nm in the case of (i) and at 287 nm in the

case of (iii). Kinetics of this reaction were measured following the increase in absorbance. First-order kinetics were independent of the wavelength observed (255 mM - 300 nM). Rate constants were determined as a function of phosphate concentration (Figure 2). Dependencies were linear following the relation for reversible platinum(II)-phosphate complexes according to

$$-\overset{|}{\underset{|}{Pt(II)}}-H_2O + \text{Phosphate} \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} -\overset{|}{\underset{|}{Pt(II)}}-\text{Phosphate} + H_2O$$

$$k_{obs} = k_{diss} + k_{ass}[\text{Phosphate}] \quad \text{for } [\text{Phosphate}] \gg [Pt(II)].$$

Rate constants k_{ass} and k_{diss} were calculated from the slopes and intercepts, respectively in Figure 2. They were $k_{ass} = 1.65\ M^{-1}min^{-1}$, $k_{diss} = 0.004\ min^{-1}$, K_{Diss}(the dissociation constant) = k_{diss}/k_{ass} = 2.4 mM in the case of (i) and $0.38\ M^{-1}min^{-1}$, $0.005\ min^{-1}$ and 13 mM, respectively, in the case of (iii).

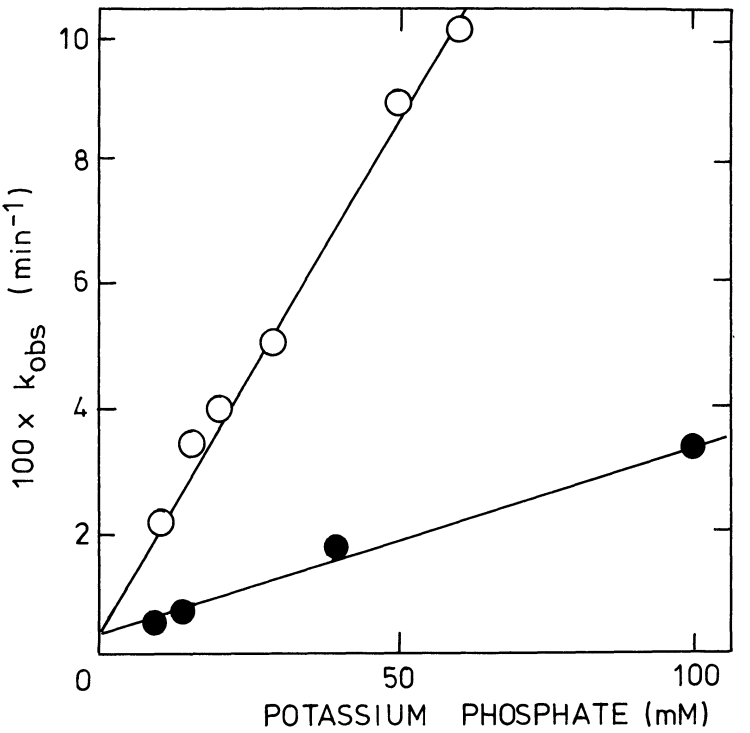

Fig. 2. Kinetics of the reaction between orthophosphate and platinum(II) complexes at pH 5.5 and 37° C. The change of absorbance at 300 nm was measured. Values of k_{obs} refer to rate constants obtained from first-order plots. Concentrations of platinum(II) complexes were 1 mM, -o- (i), -●- (iii).

Values of dissociation constants were also measured from the reaction amplitude as a function of the phosphate concentration under similar conditions as in Figure 2. Values of 3 mM for (i) and 8 mM for (iii) were calculated in reasonable agreement with those determined kinetically. The reversibility of the phosphatoplatinum(II) complexes was verified by reacting 0.5 mM salmon testis DNA with 0.05 mM phosphatoplatinum(II) complex under conditions comparable to those for Figure 1. The phosphatoplatinum(II) complexes had been prepared by reacting 1 mM plainum(II) complex over night with 10 mM potassium phosphate at pH 5.5 and 20° C. A small portion of the hyperchromic effect followed the usual kinetics seen after mixing of DNA and the platinum(II) complex and was probably due to phosphate-free complex. The majority of hyperchromicity followed a single exponential increase in absorbance with k_{obs} = 0.004 min^{1} for (i) and k_{obs} = 0.005 min^{-1} for (iii). These values are those cited above for k_{diss} and are in good agreement with the intercepts in Figure 2.

DISCUSSION

Kinetic, equilibrium and spectroscopic results show that orthophosphate binds to platinum(II) complexes. For variable conserved ligands (i) and (iii), respectively, rate constants are different for association but not dissociation of the particular phosphato complexes. The value of the association rate constant, 0.38 $M^{-1}min^{-1}$, for (iii) is comparable with that, 0.36 $M^{-1}min^{-1}$, reported under conditions of pH 6.0, 0.5 M $NaClO_4$ and 40° C (3). Dissociation rate constants observed here for the phosphato ligand have similar values in comparison to those of the chloro group (1, 3). The values of dissociation constants are of the same order of magnitude as that for dissociation of the first chloro group from (ii) (3.3 mM) (4).

The formation of phosphato complexes inhibits the reaction of the platinum(II) complexes with salmon testis DNA (Figure 1). The

particular reactions observed here are two supposed rearrangements (1, 2). The nature of the second, slow one is local "melting" of double standed DNA (5, 2) that accompanies crosslinking of DNA (1). The nature of the fast rearrangement is not known. It is not observed, if the attacking platinum(II) complex containes only a single molecule of bound water. If the other exchangeable ligand in cis-position is the chloro group or, as we find here, the phosphato group, then the fast "rearrangemet" is not detected. This explains why the concentration dependencies of the corresponding amplitudes in Figure 1 follow the values of dissociation constants that have been measured by the experiments in Figure 2. The coincidence in values of half maximal amplitudes in Figure 1 and dissociation constants provides evidence that the free energy of the fast rearrangement must be quite low.

The presence of a phosphato group in the monovalent DNA-platinum(II) complex prevents crosslinking and thus the second rearrangement unless the phosphato group hydrolyses from the DNA-platinum complex. This would give rise to a slow reaction (k_{diss} = 0.005 min^{-1}) which was not seen here. The reactions observed here in the presence of all concentrations of orthophosphate followed rate constants 0.2 - 0.5 min^{-1} for the fast and 0.02-0.06 min^{-1} for the slow rearrangements (results not shown) that were of the same order of magnitude than in the absence of phosphate (2). Moreover, it is not understood why the concentration dependence for the slow rearrangement is different from that of the fast one and why in the case of (i) and not (iii) the amplitude goes through a maximum, seemingly on cost of the rapidly declining amplitude of the fast rearrangement. All this remains unclear as long as the nature of the fast "rearrangement" is not elucidated.

The results show that orthophosphate (and possibly other forms of phosphates) can buffer platinum(II) complexes so that they would not so eagerly react with biomolecules. Such buffering would increase the fraction of a complex in chemotherapy that finally finds its biological target. Because of its higher affinity to orthophosphate, racemate 1,2-bis(4-fluorophenyl)ethylenediamineplatinum(II) sulfate (i) would be more favoured than cis-diamminediaquaplatinum(II).

We greatly acknowledge the financial support by the Deutsche Forschungsgemeinschaft grant SFB 234-86 C7, the Bundesministerium Forschung und Technik, the Herrmann Schlosser Stiftung, and the Walter Schulz-Stiftung.

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EFFECT OF *CIS*-DIAMMINEDICHLOROPLATINUM(II) ON THE NUCLEOTIDE POOL OF TREATED P388 LEUKEMIA CELLS

G. Just and E. Holler

INTRODUCTION

The Effect of cis-diamminedichloroplatinum(II) (cisDDP)-complexes on the regulatory state of P388 mouse leukemia cells has first been investigated preliminary on Ap_4A, a supposed cell growth effector (1), known already for forming a defined and non-metabolizable chelate with either cis- and trans- DDP (cis-, trans-Pt-Ap_4A)(2).

Because of very disadvantageous competition kinetics with DNA (2) an in vivo enrichment of cis-Pt-Ap_4A in treated cells has not been expected and indeed not been found. On the contrary, an analysis of nucleotides showed a more than 10-fold increase of Ap_4A in spite of the cells being proliferative inhibited in the presence of 1μM cisDDP in the medium.

At the same time also high concentrations were found for other nucleotides like cGMP, CMP, UMP, dGTP and Ap_3A in comparism to the control.

For further investigation the method of nucleotide analysis had to be optimized, providing a reproducible separation in order to confirm the above results and to trace further nucleotides like NADH, NADP, NADPH and others.

MATERIALS AND METHODS

Chemicals

Cis-diamminedichloroplatinum(II) was a gift by Degussa, Frankfurt, FRG. All radioactive tracer-nucleotides were from Amersham and all other materials of highest purity (i.e. buffer salts) from Merck, Darmstadt. Water had been conditioned by a millipore (Waters) filtration equipment.

Cell-cultures

P388 D1 Mouse Leukemia cells were seeded at 7-8x10^4/ml and cultured in the presence /absence of cis-diamminedichloroplatinum(II) at 37°C.

Extraction and cell rupture

Aliquots of the cellsuspension were taken at defined times, t_o being the point of cisDDP addition. For the first experiments (introduction) the cells were analyzed only after 48 h.

Cells were washed two times with phosphate buffered saline (PBS), and sonified. Protein (3) and DNA contents (4) were measured from aliquots. The remainder of cells was washed, pelleted and mixed with radioactive tracer-nucleotides in subpicomole amounts.

First attempts of extraction followed the protocol of Ogilvie (5), using TCA and octylamin/freon for neutralisation, or the protocol of Weinmann-Dorsch et. al. (6), using only formic acid. Better results as indicated by an improved ATP recovery were achieved by establishing the method of Shrylock et. al. (7), working with 80% methanol at 76°C which minimized nucleotide hydrolysis.

After "speedvac" evaporization and taking up in water, the probes were extracted with freon in order to remove lipids that were coextracted with methanol and were detrimental for HPLC columns. After centrifugation the aqueous phase could be directly loaded onto the HPLC-column.

HPLC-analysis

In order to achieve optimal separation of a great number of nucleotides ion exchange and reversed phase chromatography were used in sequence.

-A semipreparative ion exchange column (BioRad DEAE 5PW 7,5x 75 mm with a 35min linear gradient of 0.02-0.7M NH_4CH_3COOH at pH 8.6 and a flow of 1ml/min) was used for separation of the eluate into 5 pools.

-A reversed-phase-column (Waters C_{18}-μ-Bondapak 3.9x300 mm) with a three-step gradient of 0.6-12% methanol in 0.1M NH_4PO_4 pH 5.8: (7min 0.6%; 5min 0.6-6%; 30min 6-9.9% MeOH) was used for chromatographic analysis of each pool.

Measurements have been made on a LKB HPLC low pressure mixing equipment consisting of a controller, a pump with mixing-valve, a He solvent-conditioner and a variable wavelength UV/Vis-monitor (10μl-cell, at 254nm). The system is coupled with a Raytest radioactive monitor equipped with a splitter mixer device (2.5ml cell; 20% or 50% split; +7.5 vol Rothiszint 22 liquid scintillation coctail). UV absorbance, radioactivity and gradient forms were synchronized, digitalized and monitored as well as evaluated on an IBM compatible computer using integration and baseline correction software.

RESULTS

As already mentioned in the introduction, large differences between nucleotide levels of control and platinum treated tumor cells were observed.

An effect of cisDDP treatment could already be seen after determination of the cell protein content (Fig. 1), showing an increase during the course of cell proliferation, in comparison to the control.

Inhibition of proliferation and rise of protein content paralleled in distortion of cell shape and in an increase of cell volume that both could be observed under the microscope.

For the previous experiments amounts of nucleotides were referred to the cell numbers of the cultures. Since the cell volume increased during treatment with cisDDP, high values of nucleotide levels were observed with reference controls (in the absence of cisDDP) (see introduction). In order to avoid misleading results amounts of nucleotides are now referred to the protein content of the cell culture.

Results for ATP and dGTP (Fig. 2A and 2B) are representative for nucleoside 5'-triphosphates and -diphosphates. The levels of the controls were higher than those of Pt-treated cells. Monophosphates

exhibited similar levels for controls and platinum treated cells as shown by the example of AMP (Fig. 2C).

A pronounced effect of the treatment with cisDDP was seen in the level of cyclic AMP (Fig. 2D), showing a sharp concentration-increase of this second messenger with the beginning of cell growth and as a function of the concentration of cisDDP.

In the case of NAD/NADH and NADP/NADPH (Fig. 2E-H) the Pt-treatment of the cells is reflected by a large decrease of the oxidized species and an increase of the reduced species depending on the concentration of cisDDP. The decrease in level of NAD was not accounted for by the increase in the level of NADH.

DISCUSSION

Treatment of P388 cells with cisDDP caused remarkable disturbances in metabolic pathways and second messenger level of the cells, being reflected by inhibition of cell proliferation and rise of protein content per unit cell.

The observation of giant cells indicated that the effect of cisDDP was inhibition of mitosis rather than blocking protein synthesis. Also a decrease of protein synthesis was observed (results not shown); however inhibition of protein synthesis is not that high in order to account for the lack of mitosis.

Obviously the energetic state of the cells is highly depressed as indicated by the decreased levels of nucleoside 5'-triphosphates and -diphosphates.

Interestingly the falling level of NAD would be in accord with the hypothesis that low NAD is signalling DNA repair (8).

The drastic rise of the second messenger cAMP soon after the beginning of the treatment with cisDDP is a hint for a regulatory switch.

The observed phenomena being very complex indicate that treatment of cells with cisDDP has an impact on metabolic, energetic and regulatory events. It is hoped that an analysis of the present and of many more soluble nucleotides will substantiate the observation and will suggest routes of the antitumor mechanism of cis-platinum compounds.

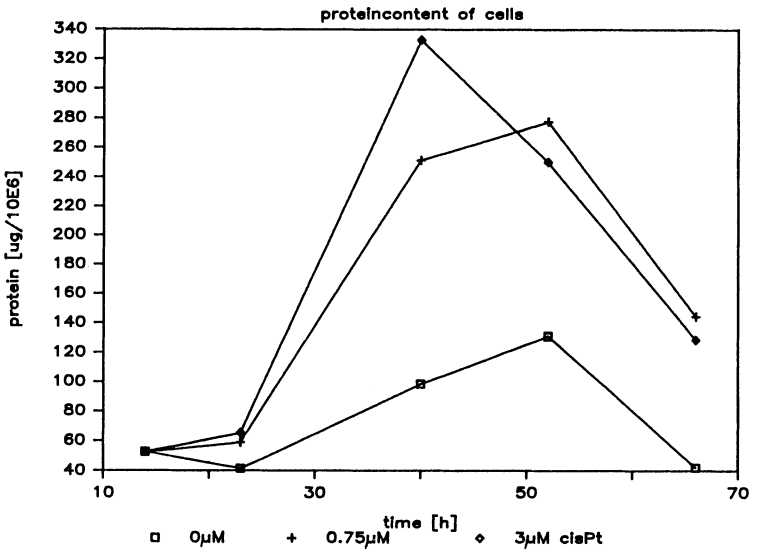


Fig.1. *Protein content of control cell cultures, and of cultures treated with 0.75μM- and 3μM cis-diamminedichloroplatinum(II)-treated P388 D1 mouse leukemia cells.(in μg protein per million cells) as a function of growth time. CisDDP was added at 14h after seeding.*

Fig.2A.

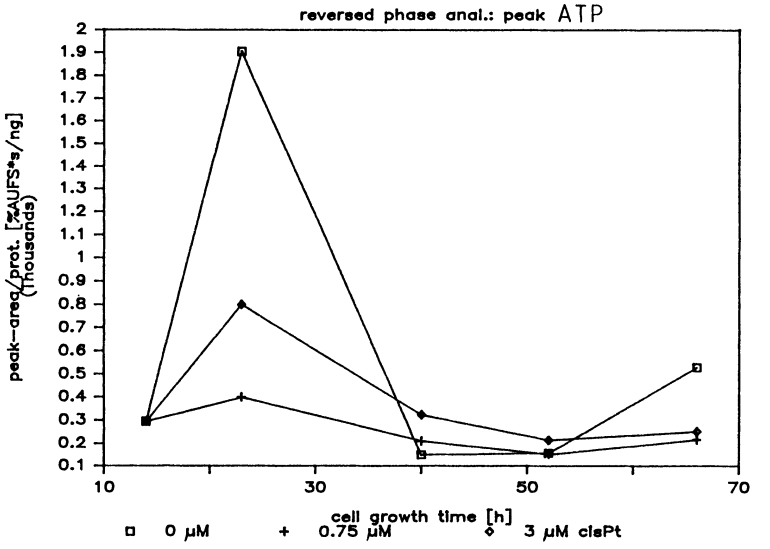
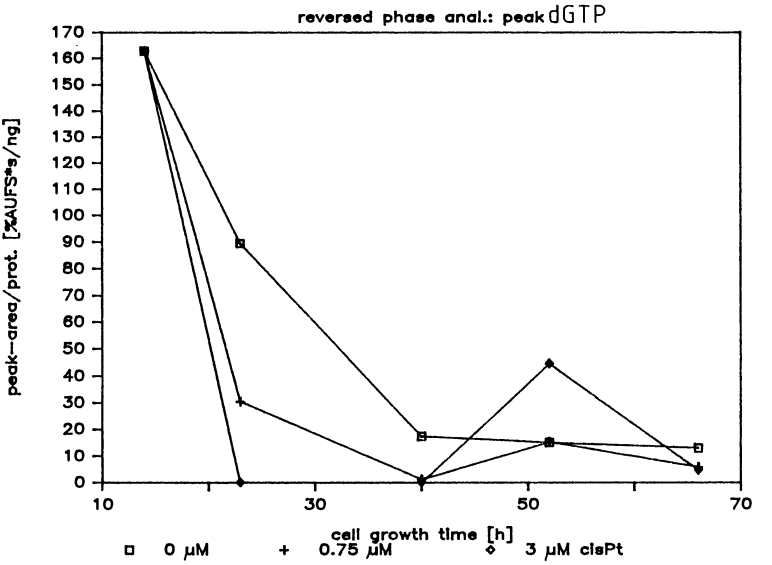


Fig.2B.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peak-area/prot. [%AUFS*s/ng]
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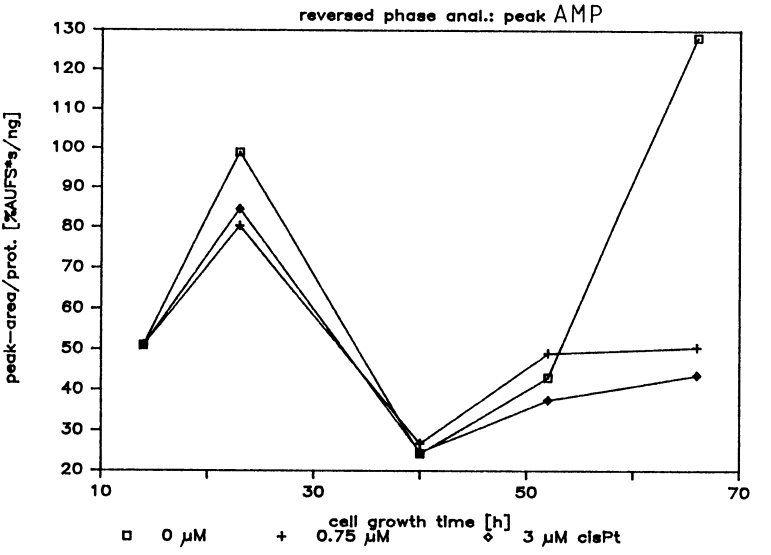
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
10
30
50
70
cell growth time [h]
□ 0 µM
+ 0.75 µM
◇ 3 µM cisPt

Fig.2C.


reversed phase anal.: peak AMP
peak-area/prot. [%AUFS*s/ng]
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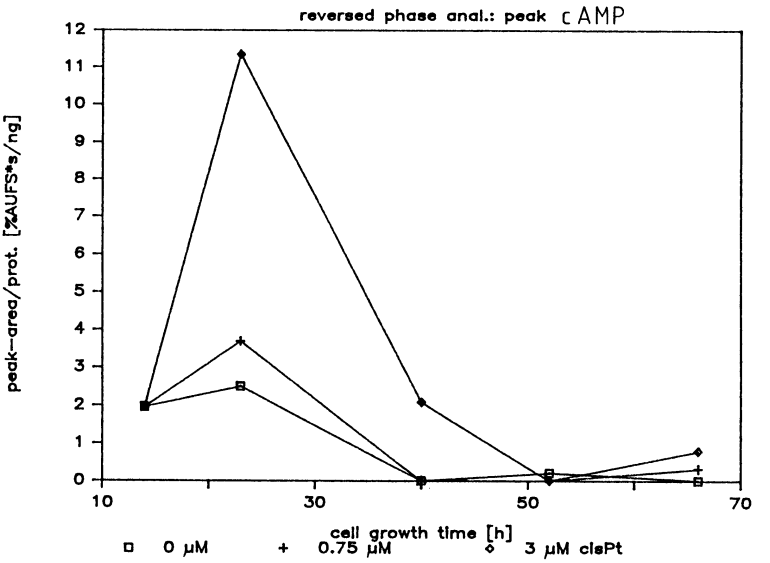
120
110
100
90
80
70
60
50
40
30
20
10
30
50
70
cell growth time [h]
□ 0 µM
+ 0.75 µM
◇ 3 µM cisPt

Fig.2D.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11
10
9
8
7
6
5
4
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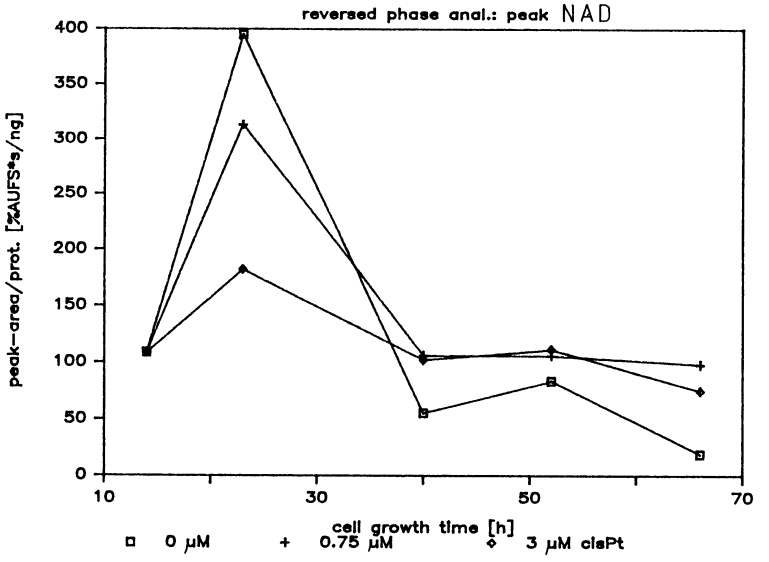
1
0
10
30
50
70
cell growth time [h]
0 µM
0.75 µM
3 µM cisPt

Fig.2E.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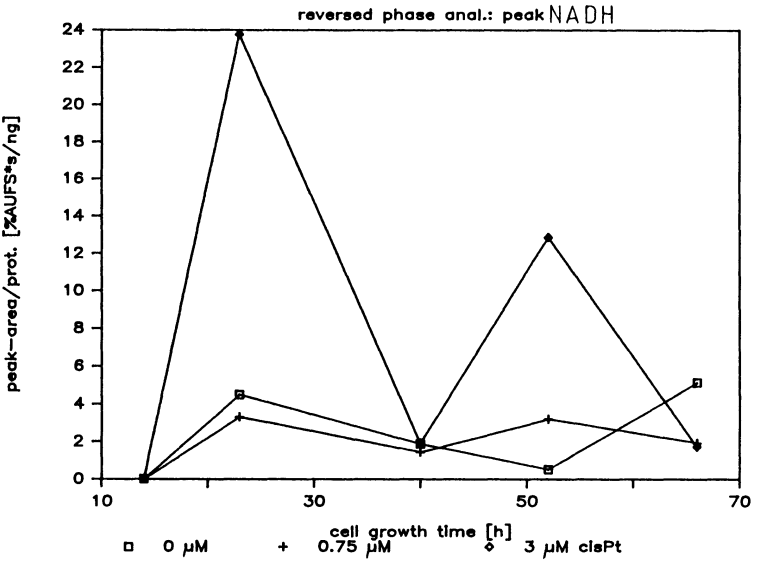
350
300
250
200
150
100
50
0
10
30
50
70
cell growth time [h]
0 µM
0.75 µM
3 µM cisPt

Fig.2F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peak-area/prot. [%AUFS*s/ng]
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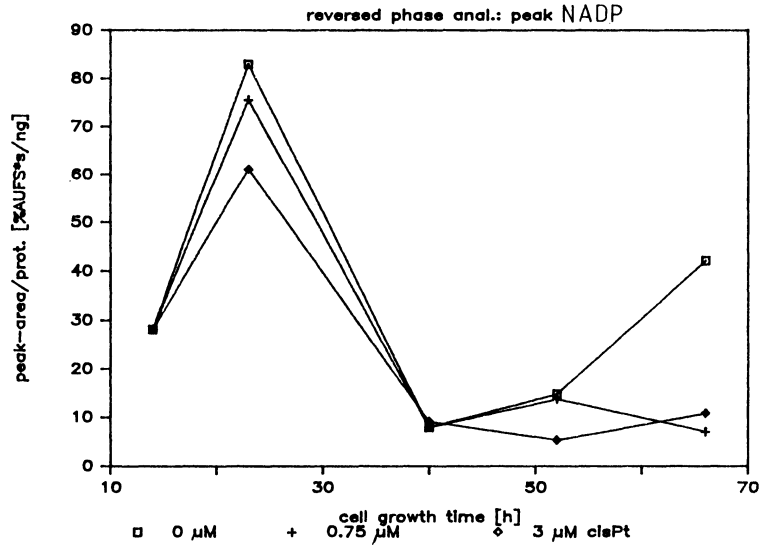
22
20
18
16
14
12
10
8
6
4
2
0
10
30
50
70
cell growth time [h]
0 µM
0.75 µM
3 µM cisPt

Fig.2G.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peak-area/prot. [%AUFS*s/ng]
90
80
70
60
50
40
30
20
10
0
10
30
50
70
cell growth time [h]
0 µM
0.75 µM
3 µM cisPt

Fig.2H

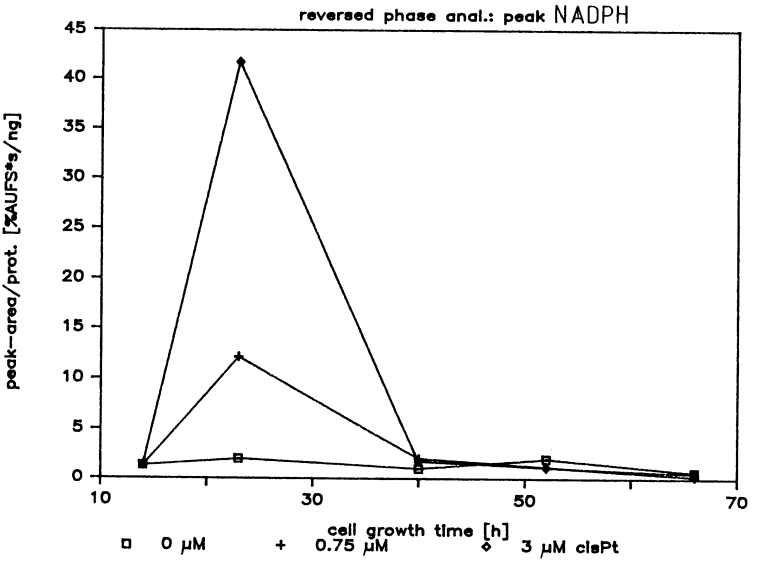

Fig.2A-H *Nucleotide levels of control, 0.75µM- and 3µM cis-diamminedichloroplatinum(II)-treated P388 D1 mouse leukemia cells versus cell growth time. The levels are measured as peak areas over the baseline of corrected reversed phase chromatograms (see methods), normalized for detector sensibility and calculated by division through the absolute protein concentration of the cell culture extract (per cent absorption units full scale at 254nm x seconds / ng protein).*

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PLATINUM CYTOSTATICS DESIGN METHOD BASED ON TOPOLOGICAL INDICES

F. Kiss, P. Tvarůžek and E. Hladká

An expert system was used previously (1) for classification of Pt complexes into classes with different magnitudes of ID 90. This system exploited fuzzy logics and enabled operations with uncertain information. Such (nondeterministic) information involve apparently the evaluation of biological effects of substances on living organisms. A database is a part of every expert system. It includes a set of complexes, every one of which being described by a n-dimensional vector of features supplemented by information on biological and/or physico-chemical behaviour of the particular compound.

In the present study we exploited a procedure classifying the complexes according their behaviour in those cases when apriori information on their properties is missing.

Topological indices (TPI) were used as features characterizing platinum complexes (2,3). For each of the complexes of non-identical sets of 38 or 52 compounds, for which solubility in chloroform (4) or values of ID_{90} (5) were published, magnitudes of 70 different TPIs were calculated (6). On their basic a correlation matrix was constructed and then 8 TPIs were chosen, which were characteristic of the whole set : Bertz complexity index (TPI 7), Kier-Hall connectivity index - order1(TPI 23), average distance sum connectivity index (TPI 49), mean square distance index (TPI 67), graph distance in-

dex (TPI 85), structure information index (TPI 113) and HV information index (TPI 139) (2,3). For each of the indices a linguistic value was assigned. It is determined by a function of membership of a triangular shape. The magnitude of the triangle base is given by the correctness of the calculation of the particular TPI. Considering the degeneration of the TPI values and the construction of the function of membership the total amount of linguistic values for individual descriptor would be : TPI 17-16, TPI 23-34, TPI 49-20, TPI 67-17, TPI 85-17, TPI 113-12, TPI 129-15, TPI 139-14. These linguastic values formed for each complex a 8-element vector and for the set of complexes studied a matrix 72x8. In the next step we sought the similarity among rows of this matrix (7). Because every of these rows represents one of the complexes, the results of this operation was a similarity graph. It vertices represent individual complexes, the edges are evaluated by numbers which indicate the similarity in the behaviour of the complexes. Because the number of vertices, with which every of 72 vertices is connected, is different, the similarity graph is desintegrated into mutually discontinnous subgraphs. Complexes which belong to vertices in the subgraphs form categories of complexes of similar properties.

Using the described method for clustering of complexes with close values of solubility in chloroform we classified correctly into 9 clusters more than 76% of all complexes. The solubilities in chloroform (in mM) of complexes belonging to the individual clusters were: for cluster No.1 (0.01, 0,01, 0.01, 0.01, 0.01, 0.01, 0.02, 0.02, 0.03, 0.06, 0.07, 0.08), cluster No.2 (0.43, 0.36), cluster No.3 (0.26, 0.14), cluster No.4 (0.05, 0.05), cluster No.5 (0.07, 0.08, 0.1), cluster No.6 (0.01, 0.08), cluster No.7 (0.008, 0.009), cluster No.8 (0.14, 0.26), cluster No.9 (0.3, 0.4).

If we classified the complexes in a similar way

using the values of ID_{90}, erroneous assignment occurred only for 6 complexes. Other molecules, i.e. 86% of all complexes were clustered correctly.

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KINETICS OF CHANGES IN THE SECONDARY STRUCTURE OF DNA INDUCED BY THE BINDING OF PLATINUM CYTOSTATICS

V. Kleinwächter, O. Vrána, V. Brabec and N.P. Johnson

INTRODUCTION

It is well established now that the reaction of the anticancer drug cis-diamminedichloroplatinum(II) (cis-DDP) with DNA is a complex process. As the primary step cis-DDP binds monofunctionally and only in a secondary reaction the bifunctional binding takes place resulting in the formation of intra- and interstrand cross-links |1-7|. Whereas there is no doubt that the monofunctional binding occurs predominantly at N-7 sites of guanine residues, there is less agreement about the nature of the adducts resulting from the bifunctional interaction |1-5|. It has been concluded that the intrastrand cross-linking is a more frequent event involving mainly two guanines and, to a lesser extent, guanine and adenine, whereas the interstrand cross-linking represents less than 1% of total platinations |3|. Kinetic studies revealed that bifunctional binding to two guanine residues occurred more rapidly than binding to guanine and adenine |1,2|. Some data have been obtained on adducts formed in the reaction of DNA with platinum compounds which do not exhibit antitumour activity, especially with the trans isomer of cis-DDP and diethylenetriaminechloroplatinum(II) chloride (dien-Pt), which serve often as model compounds in studies on the mechanism of action of cis-DDP. The monofunctional reagent dien-Pt has been reported to react predominantly with guanine bases of DNA; it reacts with adenine only at

higher Pt/base ratios |6|. For trans-DDP a two-step reaction similar to that of cis-DDP was demonstrated |2|.

In previous spectroscopic and electrochemical studies of changes in the secondary structure of DNA induced by the binding of different types of platinum compounds, including besides cis-DDP also platinum cytostatics of the second generation derived from both Pt(II) and Pt(IV), we have demonstrated that in the equilibrium state the antitumour active compounds induce in DNA conformational changes characterized as small distortions of non-denaturation nature, whereas binding of the compounds exhibiting no antitumour activity (trans-DDP and dien-Pt) led to formation of single-stranded regions in DNA molecules adjacent to the sites of platination |8,9|.

In the present study we investigated the kinetics of conformational changes induced in DNA by the binding of three platinum compounds, cis-DDP, trans-DDP, and dien-Pt, and attempted to correlate these changes with the types of adducts formed during the platination reaction.

MATERIALS AND METHODS

The experiments were carried out with calf thymus DNA prepared and characterized as described elsewhere |8|. During the isolation care was taken that the preparation should not contain any traces of denatured DNA and the amount of non-denaturation lesions should be low (as detected electrochemically |8|). Denatured DNA was prepared by 10 min heating of native DNA solution at 100 ^{o}C followed by a quick cooling in an ice bath. Platinum compounds were obtained from Lachema (Brno). DNA - Pt adducts were prepared by mixing DNA solution with solution of a Pt compound equilibrated in 10 mM sodium perchlorate (pH 5.6) in the same medium and the amount of bound platinum, expressed as r_b (molar ratio of bound platinum atoms per one nucleotide), was determined polarographically |8|. Kinetic measurements were carried out at 25 ^{o}C either with the reaction mixture or with samples from which the unbound

platinum was removed after certain periods of incubation by 30 sec centrifugation at 1500 rpm through a column of Sephadex G25.

Circular dichroic (CD) spectra were measured with a Jobin Yvon Mark IV dichrograph. Differential pulse polarographic (d.p.p.) measurements were carried out with a PARC Polarographic Analyzer, Model 174 A. The methods used for the structural analysis of DNA and determination of platinum bound to DNA were described earlier |8|.

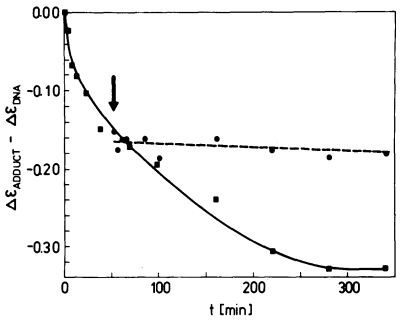
RESULTS

CD spectra

It has been shown recently that binding of platinum compounds in equilibrium state changes CD spectra of native double-helical DNA in two different ways depending on their structure and, consequently, on their biological properties |8,9|. Antitumour-active platinum compounds (i.e. compounds having a favourable therapeutic index) induce an increase of the CD band at 280 nm, whereas compounds which do not exhibit anticancer activity cause a decrease of this band. The latter property is (in distinction to the data reported previously |1|) also characteristic of monofunctionally binding platinum compounds.

The simplest pattern of changes in the CD spectra of DNA could be expected for the monofunctional binding of dien-Pt (Fig. 1). After mixing at an input ratio r = 0.08 the positive CD band at 280 nm decreased continuously. If after 50 min the unbound dien-Pt was separated (r_b = = 0.019), CD spectrum of the adduct did not change with time. However, the semilogarithmic plot of the relative spectral changes vs. time for the nonseparated mixture was neither linear nor could be resolved into a small number of components.

The binding of bifunctional trans-DDP induced similar decrease in the positive CD band, however, after the removal of unbound platinum at 50 min (r_b = 0.042) the decrease continued (Fig. 2). The semilogarithmic plot showed



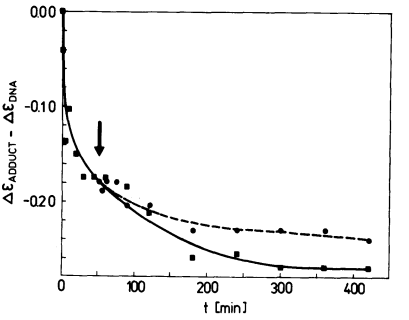
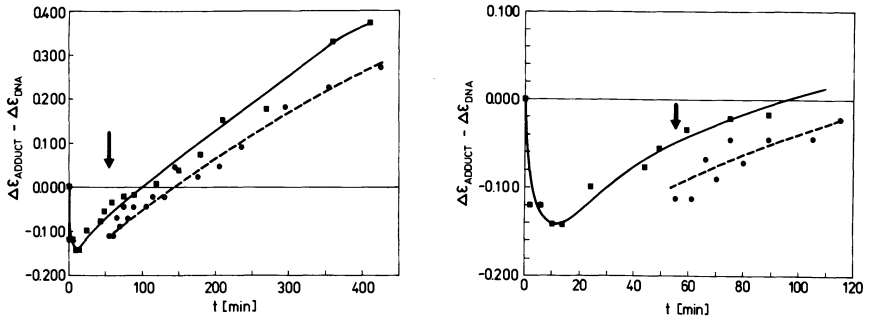


Fig. 1 Fig. 2
Changes of ellipticity at 280 nm in DNA adducts with dien-Pt (Fig. 1) and trans-DDP (Fig. 2). A mixture of DNA (0.23 mM) with platinum compounds (input r_o = 0.08) was incubated in 10 mM sodium perchlorate at 25 °C and CD spectra were recorded in the given time intervals (-■-). The other curve (-●-) was obtained for samples from which non-reacted platinum was separated after 50 min incubation (an arrow) at r_b values 0.019 and 0.042 for dien-Pt and trans-DDP, respectively.

that the changes were biphasic with apparent rate constants (k_{app}) of 0.077 and 0.003 min^{-1} (Table 1); after separation only one exponential was observed.

In contrast to dien-Pt and trans-DDP the time course of CD spectral changes induced in DNA by the reaction with cis-DDP was not monotonous (Fig. 3). After mixing (input ratio r = 0.08) the positive band at 280 nm decreased rapidly (k_{app} = 0.063 min^{-1}), reached a minimum within 5 - 10 minutes and then started to increase (k_{app} = 0.034 min^{-1}) (Table 1). If the unbound platinum was separated at any time of this early period, the positive CD band started to increase with a rate depending on the r_b value reached (Table 1).

The increase of the positive CD band was not observed for the reaction of cis-DDP with denatured DNA. The decrease of the ellipticity was monotonous and could be resolved into two exponentials (Table 1).



A B

Fig. 3 Changes of ellipticity at 280 nm in DNA adducts with cis-DDP. The conditions and curve description are the same as in Fig. 1. Part (B) is enlarged section of part (A). The r_b value at the time of separation was 0.052.

Table 1 Apparent rate constants for reaction of DNA with platinum compounds in 10 mM $NaClO_4$ at 25 °C

Ligand	Separation	CD spectroscopy		Polarography	
		$^1k_{app}$	$^2k_{app}$	$^1k_{app}$	$^2k_{app}$
	|min|	|min^{-1}|		|min^{-1}|	
cis-DDP	no	0.063	0.034	0.035	0.0025
	5	-	0.014	-	-
	50	-	0.025	-	-
trans-DDP	no	0.077	0.003	0.072	0.0037
	50	-	0.002	-	-
dien-Pt	no	*)	*)	0.0045	-
	50	-	0.000	-	-
cis-DDP + denat. DNA	no	0.081	0.007	-	-

*) not resolved

Polarography

D.p.p. analysis of changes in conformational properties of DNA during reactions with the platinum compounds investigated (not shown) was in accord with the CD spec-

troscopic observations. It was not possible, however, to distinguish the initial rapid reaction step.

Semilogarithmic plots of the kinetic curves of platinum binding to DNA obtained polarographically (plots of r_b vs. time) were resolved into two exponentials for cis- and trans-DDP while for the monofunctional dien-Pt binding only one exponential was obtained. Apparent rate constants are given in Table 1.

DISCUSSION

The observation of time-dependent changes in CD spectra of DNA during the reaction with three platinum compounds differing in their chemical and therapeutic properties is consistent with results of our earlier spectroscopic and electrochemical studies |8,9|. In equilibrium state the antitumour active compounds, i.e. bifunctional complexes with leaving ligands in cis configuration, increased both the positive CD band of DNA, and the d.p.p. peak II characterizing the presence of minor changes in the DNA secondary structure. On the other hand, monofunctional complexes or bifunctional complexes with leaving ligands in trans configuration decreased this band and induced formation of d.p.p. peak III, characteristic of denatured DNA.

It was concluded on the basis of analyses of adducts formed in the reaction of cis-DDP with DNA that monofunctional binding is the initial reaction step and bifunctional intra- and inter-strand cross-links are formed only in a secondary reaction |1-6|. The kinetics of CD spectral changes is in accord with this mechanism. In particular, in the case of the reaction of cis-DDP with native DNA the initial binding induced a decrease of the positive CD band, which was followed by the increase characteristic of the equilibrium binding |9,10|. In parallel experiments, when the unreacted platinum was removed during the initial reaction step, a rearrangement reaction was observed in the case of the bifunctional compounds, cis-DDP and trans-

-DDP, but not in the case of monofunctional dien-Pt, which apparently results in the formation of cross-links in DNA.

A finding of the biphasic kinetics obtained by the polarographic determination of bound cis- and trans-DDP deserves explanation. Since this method detects the amount of ligand molecules attached to DNA, not distinguishing between the mono- and bifunctional binding, a single exponential should be expected. The two exponentials seem to indicate that the initial reaction step of the two isomers is not a simple event. The two apparent rate constant obtained from the curves r_b vs. time may, e.g., reflect the presence of two types of binding sites on the DNA molecules for platinum binding. These results also indicate that quantitative interpretation of changes in structural parameters of DNA induced by platination in terms of rate constants is not always justified.

The kinetics of changes in the CD spectra of DNA shows that irrespectively of the type of platinum compound the initial monofunctional binding leads to formation of single-stranded regions in DNA. The different behaviour of cis-DDP and its trans isomer could be put into connection with different proportions in the formation of intra- and inter-strand cross-links. Apparently the formation of inter-strand cross-links does not induce further distortions of the DNA double helix in addition to the changes introduced by the monofunctional binding |4,8|. On the other hand, the formation of intra-strand cross-links, which are the dominant lesion in the cis-DDP binding, requires less conspicuous modifications of the DNA secondary structure |8,11| and evidently can "repair" the lesions induced in DNA by the monofunctional binding. It is probable that, in contrast to the lesions of denaturation nature, the minor disturbances in the DNA structure induced by bifunctional binding of cis-DDP are not recognised by cellular repair mechanisms. The results offer

a possible rationale for explaining the observed differences in in vivo repair of lesions induced in DNA by cis--DDP and its trans isomer |12|, which may explain the higher cytotoxicity of cis-DDP.

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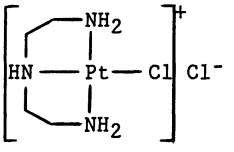
THE INFLUENCE OF THE 3'-BASE ON THE KINETICS OF THE BINDING OF [PtCl(DIEN)]Cl TO THE DINUCLEOTIDES d(GpH) (H = A,C,T)

E.L.M. Lempers, K. Inagaki and J. Reedijk

INTRODUCTION

It is generally accepted that platinum-DNA interactions are responsible for the antitumor activity of bifunctional Pt-compounds of the type cis-$PtX_2(amine)_2$ (1,2). The reaction of these Pt-compounds with DNA occurs in a two-step mechanism. The first binding step consists exclusively of binding to guanine at the N7 position. The second binding step depends on the kind of neighbouring base of the coordinated guanine. When this base is another guanine a GG-chelate will be formed. Therefore on statistical grounds one would expect 37% of the GG-chelate to be formed. However, in practice a much higher yield of the GG-chelate (65%) was found (3). Apparently, the formation of the GG-chelate cannot be explained by using statistics only. It seems that there is some kind of directing effect of Pt-compounds to GG-sequences in the DNA. This raises the question, whether there is also an influence of the other three bases (cytosine, adenine and thymine) attached to a guanine on the binding of platinum to that particular guanine. Therefore competition experiments between the several d(HpG) compounds and between the several d(GpH) compounds were set up. By using the monofunctional [PtCl(dien)]Cl the first binding step can be represented.



The results of the competition experiment between the several d(HpG) dinucleotides have already been described by Van der Veer (4) and can be summarized as :

ApG : TpG : CpG = 1 : 1.5 : 2

In other words d(CpG) has the strongest affinity for $Pt(dien)^{2+}$. In the present work the results of the competition experiment between the several d(GpH) with [PtCl(dien)]Cl will be described. By comparing this result with the data obtained by Van der Veer (4) the influence of a neighbouring base of guanine on the binding of platinum to that particular guanine will be discussed.

MATERIAL AND METHODS

Starting material

The dinucleosidemonophosphates d(GpA), d(GpC) and d(GpT) were synthesized via an improved phosphotriester method (5) and used as their sodium salts. The platinum compound [PtCl(dien)]Cl was prepared according to Watt and Cude (6).

Competition experiments

Equimolar amounts of two d(GpH) dinucleotides (about 3.10^{-6} M as determined by UV absorption) were incubated together with one equivalent of [PtCl(dien)]Cl in 300 ml doubly destilled water in the dark at pH 7.0 and 37°C. The three reactions were monitored by UV spectroscopy (Perkin Elmer EPS-3T). After two weeks the reaction mixtures were concentrated and lyophylized. In a control experiment equimolar amounts of the three d(GpH) dinucleotides were incubated together with 1.5 equivalent of [PtCl(dien)]Cl under conditions as described above. For reference purposes, the three dinucleotides were also reacted independently with a stoichiometric amount of [PtCl(dien)]Cl.

NMR measurements

To the lyophylized samples 0.5 ml D_2O (99.95 % Merck) and a trace amount of tetramethylamonium nitrate (TMA) were added. The pH was adjusted to 4.4. Proton NMR spectra were recorded on a Bruker WM 300 NMR spectrometer, at 295 K. A relaxation delay of 0.5 s and an

aquisition time of 2.0 s were used. The quantitation of the products and unreacted dinucleotides were determined by integration of the resonances of non-exchangeable nucleoside protons (d(GpA) : H1' of A; d(GpC) : H5 of C; d(GpT) : H6 of T).

RESULTS

After two weeks no change in the UV spectrum of the reaction mixtures could be observed anymore suggesting that the reactions were completed. From the ^{1}H NMR spectra of the reference experiments it was concluded that the dinucleotides all gave only one reaction product. The three reaction products all showed a downfield shift of about 0.5 ppm of the H8 resonance of guanine assuming coordination of the Pt(dien) unit to N7 of guanine. All other protons show only small chemical shift changes (< 0.2 ppm). Coordination to N7 of guanine was affirmed by the fact that the chemical shifts of the H8 protons of guanine were pH independent between pH 2.0 and 5.0. This is typical for guanine residues which are platinated at the N7 site (7). So the formation of only one reaction product, i.e. Pt(dien)[d(GpH)-N7(1)], makes the dinucleotides d(GpH) ideally suitable for investigation of the influence of the 3'-neighbouring base on the binding of platinum to guanine. By comparing the ^{1}H resonances of the reference experiments with those of the competition experiments it was concluded that the same reaction products were formed. The ratio of reacted dinucleotides was divided by the initial ratio of unreacted dinucleotides, also determined by NMR integration. The percentages of the three experiments are listed in table 1.

Table 1. Quantitation results of the competition experiments between the d(GpH) dinucleotides (The values are an average of two experiments giving an experimental error below 5%).

↓ GpA	↓ GpT	↓ GpC
45%	55%	---
50%	---	50%
---	55%	45%

So the relative binding preferences are in the ratio d(GpA) : d(GpT) : d(GpC) = 4 : 5 : 4. It should be noted that the percentages depicted in table 1., are consistent among themselves. This means that the percentages of a third competition experiment can be calculated from the other two competition experiments. In a control experiment equimolar amounts of the three dinucleotides were incubated with 1.5 equivalent [PtCl(dien)]Cl. The aromatic and H1' sugar region of the ^{1}H NMR spectrum of this competition experiment has been redrawn in figure 1.

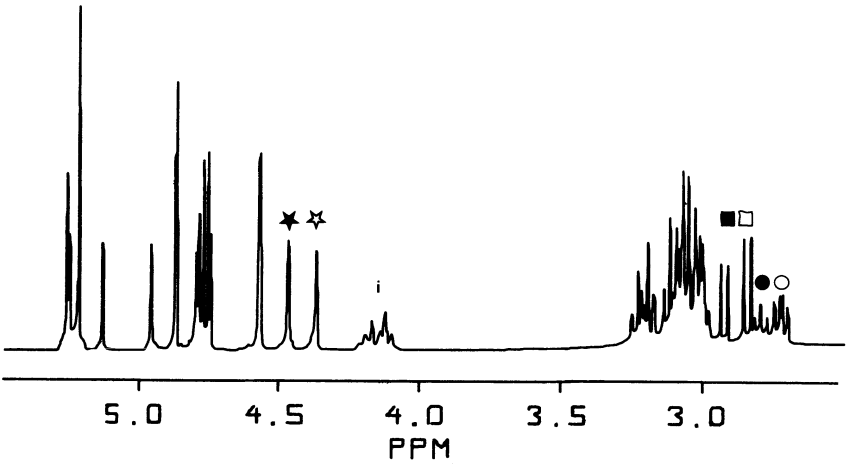


figure 1. Aromatic and H1' sugar region of the competition mixture containing d(GpA), d(GpC) and d(GpT) after the reaction with 1.5 eq [PtCl(dien)]Cl. The resonances used for the quantitation results are indicated by open and closed figures for the unbound and the bound dinucleotides, respectively (A - H1':●/○; C - H5:■/□; T - H6★/☆) . i = impurity

As expected the results obtained from this experiment are consistent with the results given in table 1.

DISCUSSION

The influence of the neighbouring 3'-base on the coordination of platinum to guanine is relatively small compared to the effect of the 5'-base in d(HpG). The difference of the influence of the H-base

on the coordination of platinum to guanine between d(GpH) and d(HpG) can be explained by assuming that in the case of d(HpG) platinum coordination is directed by the negatively charged 5'-phosphate, whereas in d(GpH) the platinum coordination to guanine is only directed by guanine itself. This 5'-phosphate directing effect has already been observed by Marcelis (8) in 5'-GMP. Consequently, the coordination of platinum to guanine in d(HpG) is much more subjected to steric effects.

One should note that results obtained with single stranded dinucleotides cannot be extrapolated directly to double stranded DNA. Nevertheless, we have shown conclusively that a neighbouring base of guanine can indeed have an influence on the kinetics of the coordination of platinum to this guanine.

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IN VITRO REPAIR OF CISPLATIN-DNA ADDUCTS BY A DEFINED ENZYME SYSTEM

J.D. Page, I. Husain, S.G. Chaney and A. Sancar

INTRODUCTION

It is now well established that DNA is the target for the cytotoxic effects of platinum anticancer drugs (1). There are molecular mechanisms that remove the Pt adducts from DNA and thus reverse the biological effects of the Pt drugs. These DNA repair mechanisms have been characterized in *E. coli*. Of particular interest is the nucleotide excision because this pathway has been shown to be the major repair pathway responsible for *cis*-DDP resistance of this organism (2). Nucleotide excision repair in *E. coli* is mediated by ABC excision nuclease (excinuclease) which is composed of three subunits, the UvrA, UvrB and UvrC proteins. The enzyme incises on both sides of the modified nucleotide(s) hydrolyzing the 8th phosphodiester bond 5' (7 bases 5') and the 4th or 5th phosphodiester bond 3' (3 or 4 bases 3') to the adduct (3). Thus it removes a little over one turn of DNA from the damaged strand. The resulting gap is filled in by DNA polymerases and sealed by ligase. The ABC excinuclease apparently recognizes the distorted helical backbone and not the nucleotide adduct that causes the distortion as it acts on adducts caused by a variety of dissimilar carcinogens and chemotherapeutic drugs (3): UV photoproducts (pyrimidine dimers and 6-4 photoproducts), psoralen, acetylaminofluorene, adriamycin, mitomycin C and *cis*-DDP adducts. Since the DNA backbone distortions caused by the various damaging agents are expected to be similar but not the same it is not surprising that the enzyme shows differential affinity. This property of the

excinuclease may be used to investigate the effects of the type of adduct (monoadduct, intrastrand crosslink, interstrand crosslink) as well as the nature of the carrier ligand (e.g. ethylenediamine *vs* 1,2-diaminocyclohexane) on the relative accessibility of Pt-DNA adducts to repair enzymes. Such information in turn may enable us to make better correlations between the therapeutic effectiveness of second generation cisplatin drugs and their carrier ligands.

MATERIALS AND METHODS

ABC excinuclease.

The enzyme was reconstituted from individually purified subunits as described previously (3). The subunits were isolated from strains that were genetically engineered to produce each of the subunits in large quantities (3,4). Helicase II (UvrD protein) was purified as described elsewhere (5), and DNA polymerase I and DNA ligase were obtained from commercial sources.

Platinum compounds.

Radiolabeled [^{3}H](1,2-diaminocyclohexane)dichloroplatinum(II)-[^{3}H-$PtCl_2$(dach) and [^{3}H](ethylenediamine)dichloroplatinum(II)[^{3}H-$PtCl_2$(en)] were prepared by Dr. Steve Wyrick of the Radiosynthesis Laboratory, School of Pharmacy, University of North Carolina. The drugs had specific activities in the range of 0.27-1.2 Ci/mmole and were better than 95% pure as judged by reverse phase high performance liquid chromatography.

DNA substrates.

We used three types of DNA substrates. First, superhelical plasmid DNA was treated with the drugs to incorporate 2-10 Pt per plasmid molecule. Second, nick translated DNA (^{32}P-dATP label) was treated with $PtCl_2$(dach) and $PtCl_2$(en) to achieve an incorporation level of about 0.05 D/N. Third, 43 bp-long oligomers containing single adduct at predetermined sites were synthesized. These latter substrates were prepared by ligating 12-mers containing GG or AG adducts with 5 other oligomers of special design. These substrates contained an internal ^{32}P label at the 6th phosphodiester bond 5' to the adduct.

Other methods.

Digestion with ABC excinuclease, analysis of the reaction products on agarose or polyacrylamide gels (3) and transformation with *in vitro* repaired DNA (6) were conducted as described elsewhere.

RESULTS

Removal of cisplatin adducts by ABC excinuclease.

The ABC excision nuclease of *E. coli* is an enzyme of many substrates. In Figure 1 the reaction products of the enzyme resulting from its action on three different substrates are analyzed on sequencing gels. The excinuclease hydrolyzes the phosphodiester bond 7 bases 5' and the phosphodiester bond either 3 or 4 bases 3' to UV photoproducts and generates 12 or 13 base-long oligomers. When DNAs damaged by $PtCl_2$(dach) or $PtCl_2$(en) were substrates, the excised fragments migrated about 2 bases slower on the sequencing gels than the 13-mers generated from UV-DNA (Figure 1A lane 1 vs lanes 3 and 4). Since it had been previously shown that ABC excinuclease incises 7 bases 5' and 3 bases 3' to *cis*-DDP diadducts to generate 12-mers (7), we reasoned that the slow migration of the excised fragment was due to the Pt adduct. Indeed when the ABC excinuclease digested DNA was treated with 10 mM thiourea before analysis on sequencing gels it was found that both $PtCl_2$(dach) and $PtCl_2$(en) adducts were removed as 12-mers as evidenced by the migration of deplatinated excision products on a sequencing gel (Figure 1B, lanes 1 and 2).

Uniform substrates containing Pt adducts at unique locations.

Site specifically platinated DNA is important for studies on the action mechanism of Pt based antineoplastic drugs (8). Having found that 12-mers containing Pt adducts migrate slower on sequencing gels and are therefore easily separable from their non-adducted counterparts, we decided to purify uniquely modified oligomers and incorporate them into longer duplexes to create unique, well-defined substrates for ABC excinuclease. The oligomers were d(CTCTCAGTCTCT) for AG diadduct and d(CTCTCGGTCTCT) for GG diadduct. When these oligomers were incubated with either $PtCl_2$(dach) or $PtCl_2$(en) a band migrating approximately 1 base slower appeared on sequencing

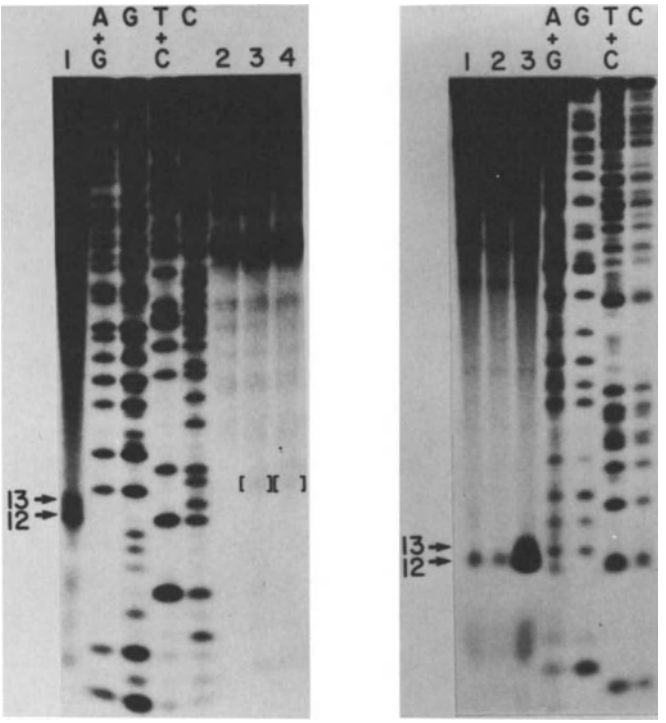

Figure 1. Excision of Pt-base adduct by ABC excinuclease. Uniformly labeled [^{32}P]-DNA was treated with $PtCl_2$(dach) or $PtCl_2$(en), digested with ABC excinuclease and then analyzed on 12% polyacrylamide DNA sequencing gels. A + G, G, T + C and C are the Maxam-Gilbert ladders used as size standards. Left, no thiourea treatment. Lane 1, UV irradiated DNA, Lane 2, undamaged DNA, Lane 3, $PtCl_2$(dach) treated DNA and Lane 4, $PtCl_2$(en) treated DNA. The 12 and 13-mers excised by the enzyme from UV irradiated DNA are indicated by arrows. The much fainter bands resulting from Pt-nucleotide excision are bracketed. Right, after thiourea treatment. Lane 1, $PtCl_2$-(dach) treated DNA; Lane 2, $PtCl_2$(en) treated DNA; Lane 3, UV irradiated DNA.

gels. The result obtained with $PtCl_2$(dach) treated d(CTCTCGGTCTCT) is shown in Figure 2. An increasing fraction of the oligomer is converted to the slow migrating species as a function of incubation time. After 50 hrs about 50% of the oligomer is converted to the modified form. Longer incubation did not increase the yield for

reasons that we cannot explain at present. Similar results were obtained with $PtCl_2$(en) modification. Considering that the slow migrating species yields a reasonably sharp band and that the sequence does not contain other reactive centers for *cis*-Pt(II) we assume that all the slow migrating DNA contains a GG diadduct at the predicted location. The adducted and non-adducted 12-mers were purified by excision of the appropriate band from the gel, electroelution of the 12-mer from the polyacrylamide and ethanol precipitation. The 12-mer with $PtCl_2$(dach) and $PtCl_2$(en) adducts at the AG sequence was prepared and purified in a similar fashion.

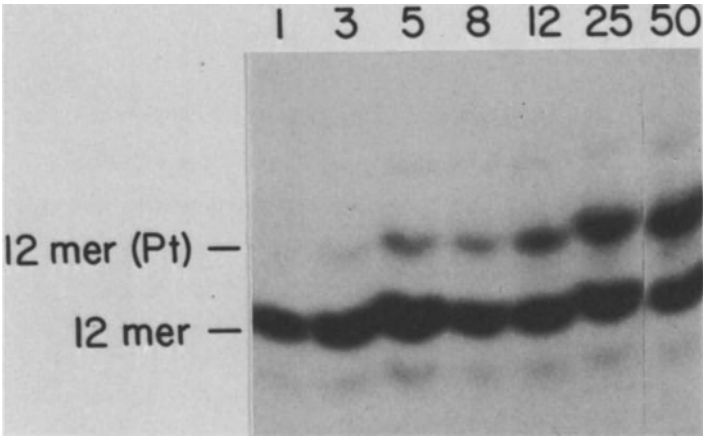

Figure 2. Preparation of 12-mer with unique Pt adduct. The p(CTCTCGGTCTCT) dodecamer at 10^{-9}M was incubated with $PtCl_2$(dach) at 10^{-8}M at 37°C for the indicated number of hrs (1-50), samples were taken and the reaction stopped with 0.5 M NaCl. The reaction products were then analyzed on a 12% sequencing gel. The slower, migrating species was presumed to be Pt[GG]-12 mer because it was converted to the fast migrating species with thiourea treatment.

The modified 12-mers were then mixed with 20-mer containing the complementary sequence in a central location and 4 other "arm" oligomers and ligated. The resulting product were 43 bp duplexes that contained 1 base overhang at each termini (to prevent end-to-end ligations). Of the 6 oligomers used in constructing the substrates only the modified oligomer was labeled at the 5' terminus with ^{32}P and therefore the final 43-bp substrates contained an internal label at the 6th phosphodiester bond 5' to the *cis*-Pt adducts.

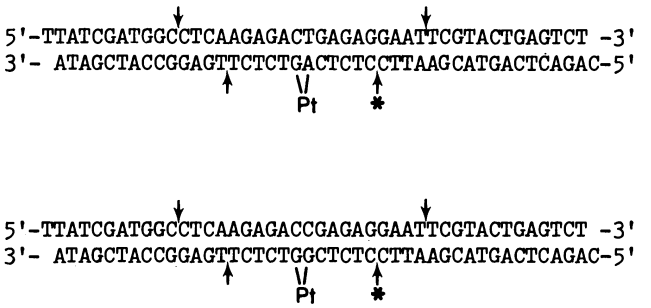

Figure 3. Synthetic substrates for ABC excinuclease. The modified [either with Pt(dach) or Pt(en)] central bottom dodecamer, was kinased with ^{32}P-ATP and ligated to the other 5 oligomers to obtain the duplexes shown here. The location of Pt adduct is indicated. The arrows represent the boundaries of the oligomers and the asteriks indicates the location of the internal ^{32}P label.

Action of ABC excinuclease on uniquely modified DNA.

Beck *et al.* (2) have previously shown that when *cis*-diammine-dichloroplatinum (II) (*cis*-DDP) treated DNA is incubated with ABC excinuclease the enzyme makes specific cuts in the DNA. From the location of these incisions relative to the potential *cis*-DDP modification sites it was concluded that the enzyme incises the 8th phosphodiester bond 5' and the 4th phosphodiester bond to GG and perhaps AG intrastrand diadducts. We have obtained similar results with DNAs modified with $PtCl_2$(dach) and $PtCl_2$(en) (data not shown). Therefore it was expected that upon incubating the uniquely modified substrates with the enzyme the nuclease would remove 12 nucleotide-long oligomers. Because of the specific location of the radioactive label in the 43-mers these 12-mers are expected to be radioactive (Figure 2) thus facilitating detection and quantitation. In Figure 4 we present the results obtained with two different Pt diadducts and two carrier ligands. From this figure the following points emerge. First, the enzyme excises Pt adducts from the synthetic substrates. Second, it appears that [AG]Pt(en) adducts are the best substrate, the order of affinity

being [AG]Pt(en) > [AG]Pt(dach) > [GG]Pt(en) > [GG]Pt(dach). The generality and significance of this observation remain to be established by further studies using longer substrates containing the adducts in different sequence context. However, it should be pointed out that the more efficient removal of the AG adducts seen in this study is in apparent contrast with the conclusion drawn from studies with randomly modified DNA which suggested GG adducts to be better substrates (7). Third, while the [AG]Pt(en) adduct is removed as a 12-mer the [AG]Pt(dach) adduct is removed in the form of 12-mer and 13-mer. Since the enzyme incises the 8th phosphodiester bond 5' to both Pt(en) and Pt(dach) adducts (data not shown), these results indicate that the enzyme on the 3' side hydrolyzes the 4th phosphodiester bond exclusively when the carrier ligand is ethylenediamine and the 4th or 5th phosphodiester bond when the carrier ligand is 1,2-diaminocyclohexane. [GG]Pt$(NH_3)_2$ adducts are removed by incision pattern similar to that of $PtCl_2$(en) adducts (7). Fourth, the synthetic 43-mers are poor substrates for the excinuclease. Under optimal conditions only 3-5% of the adduct is removed. This contrasts with 30-40% psoralen adduct removal by the enzyme from DNA fragments of the same length and essentially the same sequence.

A defined enzyme system to repair Platinum damage.

In vivo experiments (2,9) strongly sugget that nucleotide excision repair plays a major role in bacterial resistance to cisplatin. To demonstrate that the excision we observe *in vitro* does indeed result in repair in biological sense we conducted the following experiment. Plasmid DNA containing 11-13 $PtCl_2$(dach) adducts per molecule was treated with ABC excinuclease and then transfected into an *E. coli* strain deficient in excision and recombination repair pathways, CSR603 (*recA1 uvrA6*). It had been previously shown that in this strain 1.7 - 2.0 Pt adducts per molecule constituted a lethal hit (10). Treatment of the modified DNA with the purified ABC excinuclease resulted in the removal of the adducts by the enzyme and in a corresponding increase in its transforming efficiency (Figure 5). During these studies it was observed that ABC excinuclease does not turnover, i.e. only stoichiometric amounts of Pt adducts are removed. Further investigation

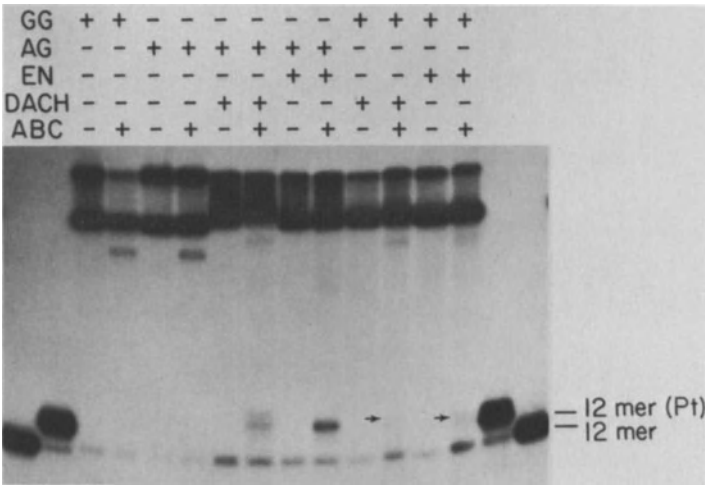

Figure 4. Removal of AG and GG diadducts from defined substrates by ABC excinuclease. The uniquely modified 43 mers containing internal ^{32}P label were digested with ABC excinuclease and the products analyzed on a 20% sequencing gel. The outer two lanes on both sides contain a Pt-modified and unmodified 12-mers as size standards. The labeling on top indicate whether the 43-mer contained GG or AG adduct, whether it was Pt(dach) or Pt(en) adduct and whether or not it was treated with ABC excinuclease. The faint band seen in all channels comigrates with the unmodified 12 mer and is due to an impurity of our 43-mer substrates. The excised [GG]Pt(en) and [GG]Pt(dach) adducts gave rise to relatively faint bands which are clearly seen in the original autoradiogram but not in this photographic reproduction and therefore their locations are indicated by arrows.

of this phenomenon led to the discovery (11) that the enzyme acts catalytically in the presence of two other *E. coli* proteins, DNA polymerase I and DNA helicase II (UvrD protein). Thus in a reaction mixture containing the ABC excinuclease (the UvrA, UvrB and UvrC proteins), DNA polymerase I, helicase II, DNA ligase and the necessary cofactors cisplatin adducts are removed from the DNA and the gap resulting from this removal is filled in and ligated to accomplish complete repair of the damaged DNA (Figure 6).

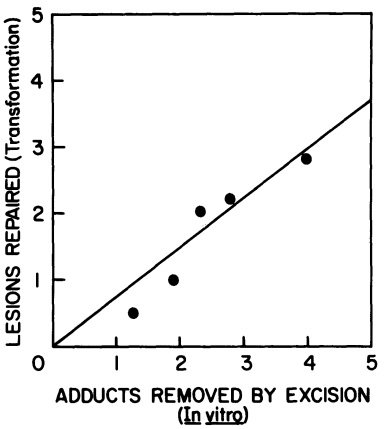

Figure 5. Removal of Pt adducts by ABC excinuclease *in vitro*, results in increased biological activity. pBR322 DNA containing 10.7 or 12.5 adducts of (^{3}H-dach)Pt was incubated with ABC excinuclease and the amount of the adduct removed was quantitated by the decrease in DNA associated radioactivity. The biological activity of the repaired plasmid was tested by its ability to transform *E. coli* to tetracycline resistance (From 6 with permission).

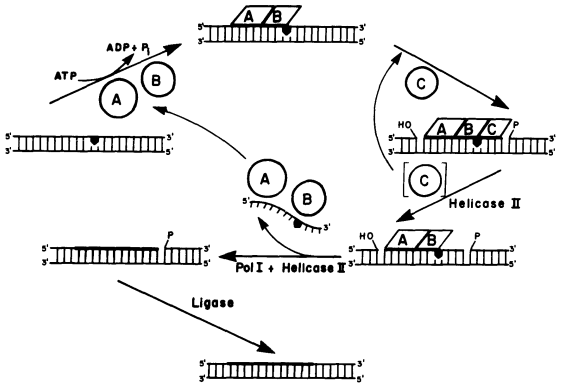

Figure 6. A defined enzyme system to repair Pt damaged DNA *in vitro*. The ABC excinuclease has 3 subunits. The A subunit binds to damaged DNA in an ATP dependent reaction, followed by the B subunit. The two together make a very stable complex at the site of Pt adduct, but do not incise the DNA. Upon addition of the C subunit dual incisions are made 7 bases 5' and 3' bases 3' to the Pt adduct. The enzyme remains attached to the incised DNA. When DNA polymerase I and helicase II are added the ABC excinuclease and the excised dodecamer are released. Polymerase fills in the resulting gap which is then sealed by DNA ligase. (From 10, with permission).

DISCUSSION

The contribution of various types of DNA adducts to the lethality of Pt drugs is not firmly established. In addition, the effect of carrier ligands to lethality must be considered in designing drugs of better therapeutic index. We have purified from *E. coli* an enzyme that repairs Pt damage of DNA. The availability of this enzyme, the ABC excinuclease, has made it possible to demonstrate the removal of specific types of DNA adducts. This *in vitro* system opens up new horizons in our study of cisplatin-DNA interactions. (1) The enzyme may be used to determine the distribution of Pt adducts in a given DNA fragment and thus enable us to determine the effect of neighboring sequences on adduct formation. Exonuclease III (11,12) or T4 polymerase 3' → 5' exonuclease (13) stopsites upon digestion of platinated DNA as well as DNA polymerase I stopsites in the polymerization reaction with single stranded template (14) have been used for this purpose. The Exonuclease III has high level of non-specific stops while the T4 polymerase 3' → 5' exonuclease does not stop quantitatively at every adduct and the polymerization assay is applicable only to single stranded DNA. We have been able to remove 80% of Pt adducts from plasmid DNA with ABC excinuclease. However, at present we obtain only partial removal from DNA fragments of about 200 bp. We hope that with further study we will be able to optimize the conditions for the enzyme and achieve quantitative removal of Pt adducts. This should enable us to determine the absolute frequency of distribution of the adducts in a given DNA sequence. (2) The ABC excinuclease may be used with defined substrates to discern the relative susceptibility to repair of various cisplatin adducts. When randomly damaged DNA is used as substrate the majority of excinuclease incisions correspond to the cuts for removal of GG intrastrand diadducts (7). Yet, the results presented in this paper indicate that AG diadducts are removed more efficiently than GG diadducts. Thus it appears that the low cutting at AG sequences compared to GG sequences observed with randomly damaged DNA is due to the lower frequency of formation of Pt adducts at AG sequence. A similar conclusion was reached by analysis of T4 DNA polymerase digestion pattern of terminally labeled-platinated DNA (13). (3)

We find that the carrier ligands affect the relative rate of repair of the Pt-base adducts by ABC excinuclease. Thus, the enzyme may be used as a reagent in characterizing second generation Pt drugs. It is interesting to note that *trans*-DDP, which is therapeutically ineffective, is relatively non-toxic to *E. coli* and its DNA adducts are poor substrates for ABC excinuclease (9). (4) Finally, ABC excinuclease may be used to quantitate the Pt adducts in specific sequences in the human genome, e.g., oncogenes. DNA from cisplatin treated cells or individuals is digested first with a restriction enzyme and then with ABC excinuclease, separated on an alkaline agarose gel and probed with the sequence of interest. The decrease in intensity of the specific band on Southern gels as a result of ABC excinuclease treatment should be a measure of the level of modification by cisplatin of that specific sequence. In conclusion, using ABC excinuclease with new HPLC analytical methods for separating the various cisplatin adducts (15,16) it should be possible to study the mechanism of cisplatin resistance in both model systems (17,18) and in clinical setting (19,20).

ACKNOWLEDGMENT

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IMPORTANCE OF DNA SEQUENCE IN THE REACTION OF d(ApG) AND d(GpA) WITH *CIS*-DIAMMINEDICHLOROPLATINUM (II)

A. Rahmouni, A. Schwartz and M. Leng

INTRODUCTION

Numerous studies support the concept that DNA is the likely target for the antitumor drug cis-diamminedichloroplatinum (II) (cis-DDP) (1-3). Most of the adducts formed in the reaction of cis-DDP and DNA have been identified (4-6). Two major adducts arise from an intrastrand cross-link between two adjacent guanine residues and between adjacent adenine and guanine residues. Minor adducts arise from intrastrand cross-links between two guanine residues separated by one base and from interstrand cross-links between two guanine residues.

The nature and the amount of adducts have been deduced after complete enzymatic digestion of platinated DNA (4-6). An important question is to know whether among the DNA potential binding sites, some of them are more or less reactive with cis-DDP. Several experiments are in favor of a preferential binding of cis-DDP to $(dG)_n \cdot (dC)_n$ (7-11). Nevertheless, digestion of platinated DNA restriction fragments with exonuclease III has revealed that some d(GpG) are less reactive than expected (11-12). The sequence specificity of cis-DDP binding to the BamHI-SalI 275 base pair fragment from pBR322 was also mapped by digestion with the 3'-5'exonuclease activity of T4 DNA polymerase (13). All the d(GpG) and all the d(ApG) but one in the sequence d(TpGpApGpC) were reactive with cis-DDP.

We have undertaken a systematic study of platinated DNA restriction fragments by means of the 3'-5' exonuclease activity of T4 DNA polymerase. We show here that the reactivity of d(ApG) and d(GpA) with cis-DDP are dependent upon the DNA sequence.

MATERIALS AND METHODS

Three plasmid DNAs, pBR322, pUC19 and pDLP13 (a gift of Dr. D. Pulleyblank (14)) were prepared as described in ref. 15. Stock solutions were made in 10 mM $NaClO_4$, 1 mM phosphate buffer pH 7.5.

The restriction enzymes and alkaline phosphatase were purchased from Boehringer-Mannheim, T4 polynucleotide kinase from Bethesda Research Laboratories, T4 DNA polymerase from Biolabs. Enzyme buffers were those recommended by the suppliers. Ultrapure agarose was from Bethesda Research Laboratories and electrophoresis-grade acrylamide and bis-acrylamide from Bio-Rad. cis-DDP was a gift from Dr. J.L. Butour.

Obtention of DNA restriction fragments, 5'-end labeling, reaction of cis-DDP and DNA, digestion of platinated samples with T4 DNA polymerase and electrophoresis were performed as previously described (13).

RESULTS AND DISCUSSION

In order to determine the DNA binding spectrum of cis-DDP, the experimental scheme was as follows. A DNA restriction fragment radioactively labeled at the 5'end was reacted with cis-DDP. The extent of cis-DDP modification was such that on average 1-1.5 platinum residues were bound per strand. The platinated fragment was then digested with T4 DNA polymerase under conditions in which the enzyme functions as a 3'-5' exonuclease. The resulting fragment (before and after removal of bound platinum by incubation with KCN (16)) were resolved on a Maxam-Gilbert sequencing gel along with the corresponding Maxam-Gilbert sequencing ladders of the same unplatinated restriction fragment. Previous results (see Fig. 4 in ref. 13) and Fig.1 in this paper show that the enzyme is stopped (or at least is slowed down) by the presence of bound platinum. In the absence of bound platinum, the DNA fragment is completely digested (13, 17).

In a first set of experiments, we have studied the (185-375) EcoRV-BamHI (labeled at the BamHI site) fragment from pBR322, the (375-651) BamHI-SalI (labeled at the SalI site) fragment from pBR322 and the (447-628) HindIII-PvuII (labeled at the HindIII site) fragment from pUC19. Because of the gel resolution, about 524 bases out of

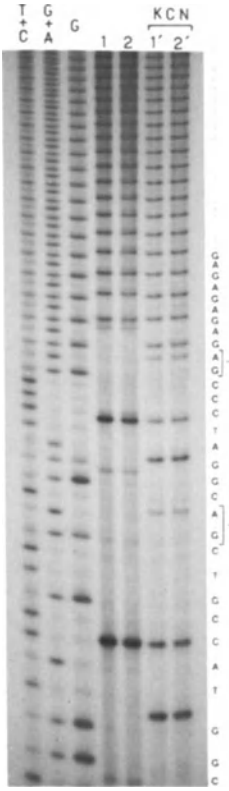

Fig. 1 : Autoradiogram of a 10 % polyacrylamide /8M urea electrophoresis gel showing results for T4 DNA polymerase digestion of platinated EcoRI-HindIII (93-base pairs) fragment from pDLP13. The fragment is($5'-^{32}P$)-end-labeled at the EcoRI extremity. Electrophoresis was carried out at 40 W constant power for 3 hours. cis-DDP modified fragment at molar ratio cis-DDP over nucleotide equal to 0.006 (lane 1), 0.012 (lane 2). In lanes 1' and 2' the two samples have been incubated in KCN before electrophoresis. The lanes labeled T+C, G+A and G are Maxam-Gilbert sequencing reactions of the same unplatinated restriction fragment.

647 have been analyzed. The results are the following :

Sites	Enzyme stopping sites
34 d(GpG)	34
27 d(GpA)	0
38 d(ApG)	33

Enzyme stopping sites were observed at all the d(GpG). However, the band intensity corresponding to d(GpG) in the sequence GTGGTC at position 335 in pBR322 is very weak as compared to the d(GpG) in the same GTGGTC sequence at position 418 and more generally to the other d(GpG). No stopping sites were observed at the d(GpA) in good agreement with data from complete enzymatic digestion studies

of platinated DNA (4-6).

Most of the d(ApG) are reactive with cis-DDP. Generally, the band intensity of these sites is smaller than that of d(GpG) sites. Assuming that the band intensity on the gel corresponds to binding site frequency, binding of cis-DDP occurs less frequently at d(ApG) than at d(GpG), in agreement with the enzymatic complete digestion studies (4-6). The sequences surrounding the five unmodified d(ApG) are respectively TTGAGCA, ATGAGCC, GCGAGCC, ACGAGCC and GTGAGCT (a d(ApG) is modified in the sequence ATGAGTG). We conclude that d(ApG) in the sequences pyGAGCpy and pyGAGCA does not react (or very weakly) with cis-DDP. However, since the enzyme is slowed down but not completely stopped by the adducts, the formation of another adduct not revealed by the enzyme is not excluded.

In a second set of experiments, we have studied the HindIII-EcoRI generated 93-base pair restriction fragment from pDLP13. This fragment contains a $d(ApG)_{22}$ insert. The results are shown in Fig.1. As expected, the 2 d(GpG) are stopping sites. In the $d(ApG)_{22}$ insert, all the d(ApG) and none of the d(GpA) are stopping sites. However, 2 d(GpA) react with cis-DDP. One is located at the junction (CCGAG) between the insert and the rest of the fragment and the other in the CGAC sequence is at 10 bases from the 5'side of the insert. The same CGAC sequence at position 635 in pBR322 is not a T4 DNA polymerase stopping site. It is surprising that in this restriction fragment, two d(GpA) are reactive with cis-DDP while none of them are reactive in the three other fragments. Several studies suggest that homopurine-homopyrimidine stretches are in an alternate (non-B, non-Z) conformation (18-20). It might be that contiguous regions to the insert in the restriction fragment are forced into a new conformation in order to minimize the free energy of the interface which makes possible the reaction between d(GpA) and cis-DDP. There are now several examples illustrating this point (21 and references herein).

In conclusion, the sequence specificity of platinum binding was mapped by T4 DNA polymerase digestion of several restriction fragments. Most but not all the d(ApG) react with cis-DDP. On the other hand, a few d(GpA) react with cis-DDP. It is suggested that this is due to unusual structures of the potential binding site induced by

the adjacent bases or by some sequences further away.

ACKNOWLEDGMENTS

We are indebted to Dr. B. Malfoy and Dr. J.M. Malinge for helpful discussions. We thank Dr. D. Pulleyblank for the gift of the plasmid pDLP13 and Dr. J.L. Butour for the gift of cis-DDP.

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CHANGES IN UV-ABSORBANCE ARE DUE TO SLOW AND FAST CONFORMATIONAL REARRANGEMENTS DURING DNA-PLATINATION

W. Schaller and E. Holler

INTRODUCTION

Kinetics of DNA platination involve at least two conformational rearrangements (1). The fast one is observed by ethidium bromide-dependent fluorescence, and its nature is unknown. The slow one refers to partial melting of DNA and is seen by circular dichroism and Tb^{3+}-dependent fluorescence. We report here that the rearrangements are responsible for UV-hyperchromic effects known to accompany DNA platination (2).

MATERIALS AND METHODS

Salmon testis DNA was purchased from Sigma and dialysed against several changes of a solution of 10 mM KNO_3 pH 5.5 before use and stored at -20° C. The racemate of 1,2-bis(4-fluorophenyl)ethylenediamineplatinum(II) sulfate (i) was a gift of Dr Schönenberger/Regensburg, cis-diamminedichloroplatinum(II) (ii) was a gift of Degussa/Frankfurt, and all other chemicals were of highest available grade from Merck/Darmstadt. Compound (i) reacted in solution as its diaqua complex. Cis-diamminediaquaplatinum(II) (iii) was prepared by the reaction of (ii) with $AgNO_3$ in a 1 : 2 stoichiometry over night at 37° C. All reaction solutions were pH 5.5 at 37° C and contained 10 mM KNO_3. Concentrations of DNA were determined spectrophotometrically and are given in concentration of nucleotide bases as described (3). Platination of DNA was carried out directly in photometric quartz cells and was continuosly under spectrophotometrical or fluorometrical observation (in the presence of ethidium bromide) as described (1).

RESULTS

Platination of DNA exhibited a hyperchromic effect in the UV-range 255 nm - 310 nm. Kinetics were the sum of two superimposed exponentials similarly as in previous cases (1). Rate constants and reaction amplitudes of the exponentials were measured as a function of DNA concentration (Figure 1). Reaction amplitudes incraesed proportional to DNA concentrations, while values of the rate constants became independent at high concentrations in the case of the faster of the two superimposed reactions. Rate constants of the slow reaction were independent over the whole range of concentration investigated. In the case of (i) (Figure 1), the rate constant for the slow reaction was 0.04 - 0.06 min^{-1} and that for the fast reaction in the plateau 0.5 min^{-1}. The initial portion of the concentration dependence was interpreted to follow a second-order rate constant of 1.5 x 10^3 $M^{-1}min^{-1}$. The corresponding values

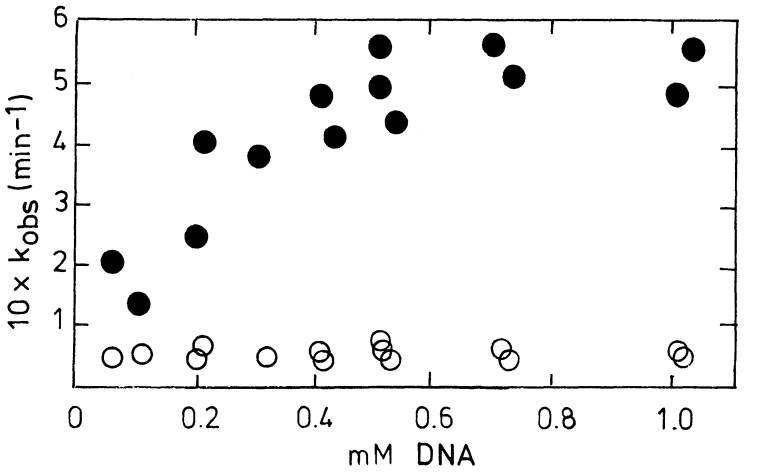

Fig. 1. Reaction of salmon testis DNA with 1,2-bis(4-fluorophenyl)-ethylenediamineplatinum(II) sulfate (i). Rate constants of the two superimposed exponentials as a function of DNA concentration. The ratio of concentrations of platinum to DNA nucleotides was 0.1. Open circles, the slow reaction. Filled circles, the fast reaction.

for (iii) were 0.02 - 0.03 min^{-1}, 0.25 min^{-1} and 1.6 x 10^3 $M^{-1}min^{-1}$, respectively. In contrast, the reaction of cis-diammineaquachloro-platinum(II) (prepared by 12 h incubation of 2 mM (ii) at 37° C (1)) was monophasic following a first-order rate constant of 0.005 min^{-1}.

The monocyano complex of (i) (prepared by photometrically controlled reaction of (i) with cyanide in a 1 : 1 stoichiometry; observation of the cyanylation at 300 nM) was spectroscopically inert when reacting with salmon testis DNA. Rate constants obtained for (iii) from UV measurements at 280 nm and those obstained from ethidium bromide dependent fluorescence (1) are compared in Figure 2 for the fast exponential time dependence. They are virtually indistinquishable.

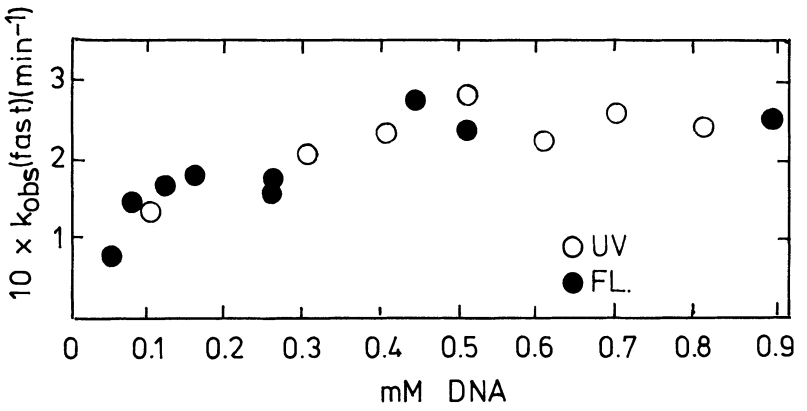

Fig. 2. Comparison of rate constants for the fast exponential reaction observed either by UV absorbance at 280 nm or by ethidium-dependent fluorescence according to (1). The concentration ratio of cis-diamminediaquaplatinum(II) (iii) to DNA nucleotides was 0.1.

The kinetic results were independent of the concentration ratio of platinum(II) complexes to DNA nucleotides up to the value 0.15.

The dependence of the kinetic parameters was investigated as a function of the spectral wavelength. Rate constants were independent of the joice of wavelength. The dependence for the reaction amplitudes is shown in Figure 3 for compound (i) and in Figure 4 for compound (iii). The position of maxima is different for the two reactions and for the two compounds. Also shown is the superposition of amplitudes of both reactions that may be compared with the net difference spectra after the reaction of DNA with the two compounds (upper trace in Figure 3 for (i), see (2) in the case of (iii)).

Results of experiments aimed at an elucidation of the nature of the fast and the slow reactions were as follows. Platination of poly d(GC) by (iii) followed again a superposition of two exponen-

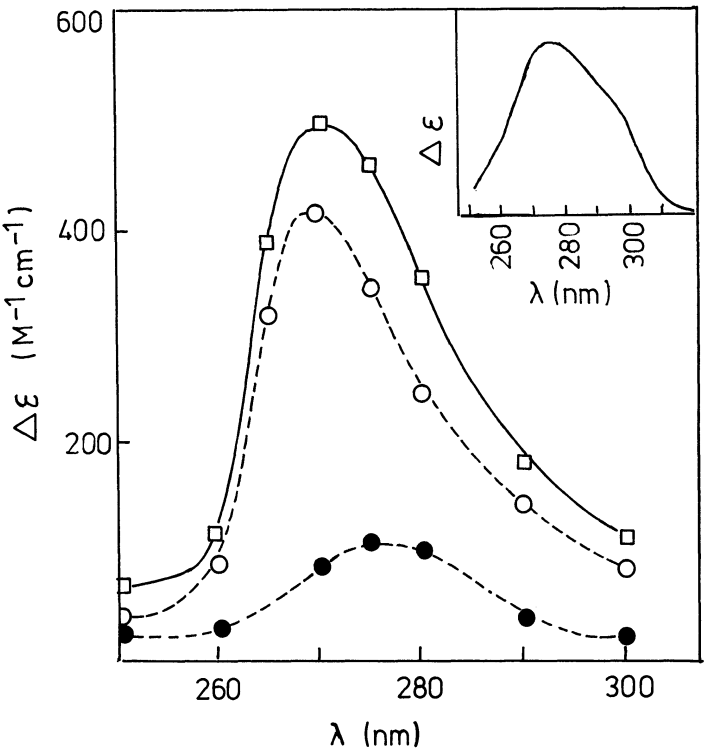

Fig. 3. Amplitudes of the slow (-●-) and fast (-o-) absorbance changes accompanying the reaction of racemate 1,2-bis(4-fluorophenyl)-ethylenediamineplatinum(II) sulfate (i) 0.05 mM with salmon testis DNA (0.5 mM). Superposition of the two amplitudes (-□-). Difference spectrum scanned after DNA platination in the inset.

tial reaction kinetics with rate constants 0.012 ± 0.002 min^{-1} for the slow and 0.12 ± 0.02 min^{-1} for the fast component (0.0125 - 0.2 mM DNA nucleotides and a concentration ratio of platinum(II) to nucleotides of DNA of 0.1. Observed wavelength 280 nm. Platinum(II) complex (iii)). The qualitatively very similar kinetics compared with that of salmon testis DNA show that the observed reactions not necessarily involve only adjacent guanine residues. The reaction of phage fd 109 single-stranded DNA exhibited first-order kinetics with a rate constant of 0.24 min^{-1} (0.25 - 0.8 mM DNA and a concentration ratio of 0.1. Observed wavelength 280 nm. Platinum(II) complex (iii)) indicating that the slow but not the fast reaction was due to the presence of double-stranded DNA. The appearance of single-stranded DNA during the reaction of salmon testis DNA (0.015 - 0.09 mM)

with (iii) at a concentration ratio of 0.1 (platinum to nucleotides) was indicated by an enhancement of Tb^{3+} fluorescence according to (4) and following after an initial lag-phase of several minutes a first-order rate constant of 0.032 +_ 0.014 min^{-1} (the concentration of Tb^{3+} was 0.01 mM). The requirement for the fast absorbance change of the polymerized nucleotide chain seems indicated by the reactions of either dGMP or GMP with (iii) that follow the bimolecular rate constant of 41 $M^{-1}min^{-1}$ of a concentration range of 0.05 - 10 mM nucleotides investigated and a concentration ratio of 0.1 for platinum to nucleotide (observed wavelength 280 nm).

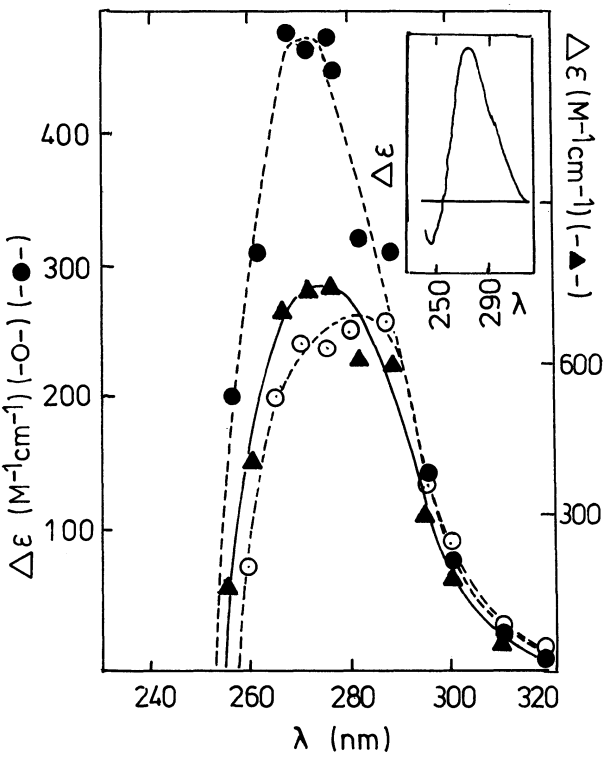

Fig. 4. Amplitudes of the slow (-●-) and fast (-o-) absorbance changes accompanying the reaction of cis-diamminediaqua-platinum(II) (iii) (0.05 mM) with salmon testis DNA (0.5 mM). Superposition of the two amplitudes (-▲-). Difference spectrum scanned after DNA platination in the inset.

DISCUSSION

We have recently described the reaction of platinum(II) complexes with DNA to follow a four step kinetic mechanism. Two of the steps were attributed to rearrangements, the slower one following DNA crosslinking and probably resembling melting of DNA at the location of platinum binding. The fast rearrangement followed the bimo-

lecular formation of monofunctional DNA-platinum(II) adducts; its nature is not known (1).

The interpretation of the slow conformational rearrangement as local melting of platinated DNA is confirmed here by the correlation of its time dependence with the appearance of single-stranded DNA. It is also consistent with the observed lack of this reaction in the case of single-stranded phage fd 109 DNA. The change in secondary structure of DNA has been assumed to give rise to the observed hyperchromicity absorbance band at 270 nm (2), and this is confirmed by the present results for cis-diamminediaquaplatinum(II) (Figure 4). However, the situation seems different for platination of DNA with racemate 1,2-bis(4-fluorophenyl)ethylenediamineplatinum(II), where this band has shifted to higher wavelengths and has relatively low hyperchromicity (Figure 3).

Hyperchromicity has been attributed to a second source: the change in electron distribution of nucleic acid bases (2); and this contribution to hyperchromicity is centered at 295 nm (2). By comparison with the present results, this component of hyperchromicity has its source in our "fast rearrangement" (Figure 4). This reaction is also ascribed to provoke the decrease in intensity of the ethidium bromide-dependent fluorescence (1) as supported by the present results (Figure 2).

A change in electron distribution of nucleic acid bases that would give rise to the hyperchromicity at 295 nm (2) is likely to follow bimolecular reaction kinetics und thus be identical with the reaction forming monofunctional DNA-platinum(II) adducts. Moreover, such interpretation would be likely to predict very similar behaviour for platinum(II) complexes carrying different conserved ligands as for compounds (i) and (iii). However, the spectral behaviour is quite different in these two examples, and the hyperchromicity at 295 nm does not follow a second-order kinetic mechanism. We are therefore declined to assume that the interpretation must be more complicated than outlined previously (2) though it seemed to fit the results of platination of mononucleotides. For the same reasons we believe that the absorbance ratio $\Delta A_{270}/\Delta A_{295}$, as proposed, is not a good index for the change in secondary structure of DNA induced by binding with platinum(II) complexes.

We are thus left with the problem of assignment of our "fast rearrangement". As we have shown, the reaction appears to accompany bimolecular attack of guanosine monophosphates by platinum(II) compounds. If it is not the primary attack itself but a reaction that joins it on the same nucleotide molecule, it must be fast compared with the attack in order to explain the dependence on nucleotide concentration. Since the reaction follows first-order kinetics for platination of polynucleotides (single- or double-stranded), we conclude that in this cases the primary attack and the reaction under consideration are separated by a rearrangement which is also unknown. The requirement that the second leaving group must be water (and neither chloride, phosphate or cyanide) seems to indicate that the observed hyperchromicity of the "fast rearrangement" is due to coordination of this second bond with some residue on the attacked nucleotide molecule. This coordination product would be readily opened in a subsequent reaction to yield DNA crosslinking. Clearly these speculations require further clearification, and work is in progress in this direction.

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VISUALIZATION OF CISPLATIN-DNA AND CARBOPLATIN-DNA ADDUCTS IN TISSUES AND CULTURED CELLS

P. Terheggen, A.C. Begg, B. Floot, J. Emondt and L. Den Engelse

INTRODUCTION

We are interested in the contribution of cisplatin- (cis-diamminedichloroplatinum, cDDP) and carboplatin- (cis-diammine (1,1-cyclobutanedicarboxylato)-platinum(II), CBDCA) induced DNA modifications to the cytotoxic and other biological effects of these drugs. An antiserum against in vitro cDDP modified DNA was therefore applied in an immunocytochemical double peroxidase assay. It proved to be possible to visualize cDDP-DNA and CBDCA-DNA binding both in tissues and cultured cells (1).

MATERIALS AND METHODS

Animals and cells. Inbred C3H mice (20-40 g) received 0-8 mg/kg cDDP and were killed after 6 hr; 6 mg/kg cDDP, killed after 6 hr-162 days; 200 mg/kg CBDCA, killed after 4 hr (2 mice for each combination). Control mice were injected with 1 ml solvent (0.14 M NaCl). Agents were given by i.p. injection. C3H mice with s.c. growing transplantable tumours (RIF1, testis and mammary tumour) were given 0-16 mg/kg cDDP and killed after 2-25 hr. Mouse RIF1 and chinese hamster CHO tumour cells were grown as monolayer cultures in Ham's F10 medium plus 10% foetal calf serum. Human buccal epithelial (HBE) cells were collected by scraping from healthy coworkers. Cells were incubated for 1 hr with 0-40 µg/ml cDDP, postincubated for 3 hr without cDDP (except in case of HBE cells), and cytospin slides were made. Cell killing was determined by colony formation.

Immunocytochemical assay. The preparation of the anti-cDDP-

DNA antiserum was carried out essentially as described by Poirier et al. (2). For the peroxidase assay, existing protocols (ref. 3, tissues; ref. 4, cells) were adapted. Nuclear staining intensity was measured microdensitometrically.

RESULTS

Normal tissues. Nuclear staining was absent from tissue sections of untreated mice, or from sections from cDDP-treated mice when the anti-cDDP-DNA antiserum was replaced by NRS or had been pre-incubated with cDDP-DNA. Specific nuclear staining was observed in liver, kidney, pancreas, muscle, brain, small intestine and testis. Minimal doses of cDDP after which cDDP-DNA binding could be observed were 0.1 mg/kg (pancreas), 0.5 mg/kg (liver, kidney, heart), 1 mg/kg (muscle, hinds), 2 mg/kg (brain, small intestine, spleen) and 4 mg/kg (testis). Staining intensity correlated positively with dose. Heterogeneity of cDDP-DNA binding within one tissue was seen in the kidney, testis, and small intestine. cDDP- or CBDCA- induced nuclear staining intensity was much higher in the renal cortex than in the medulla. Nuclei from tubules, adjacent to renal corpuscules, showed a very high staining intensity. These nuclei probably belong to the most proximal part of the proximal tubules. Nuclear staining in duodenal epithelial cells was higher than in other duodenal cells. In testis, cDDP-DNA binding was almost restricted to interstitial cells.

Persistence. After 6 mg/kg cDDP, DNA lesions could be visualized for up to 9 days (testis, spleen), 27 days (pancreas, small intestine, muscle), 81 days (liver, brain, heart) or 162 days (kidney). In general, nuclear staining persisted longer in tissues with a relatively high initial level of nuclear staining (kidney, liver, heart). Fig. 1 indicates a high rate of adduct loss in liver and kidney in the first days post treatment and a very slow rate thereafter.

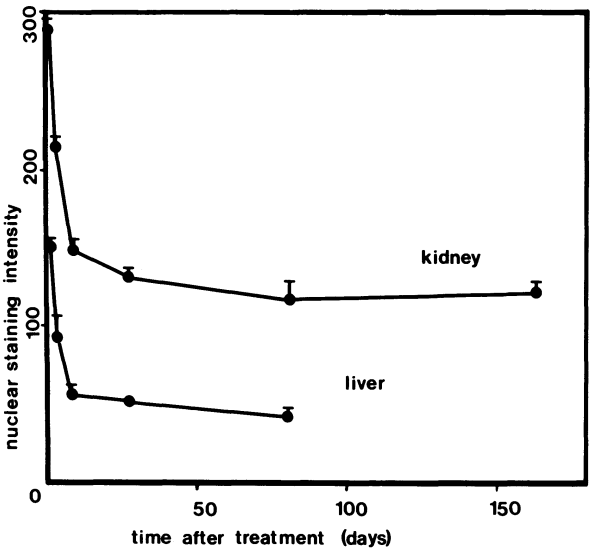

Fig. 1. Nuclear staining intensity, indicating cDDP-DNA binding, in murine kidney and liver at different time-points after an i.p. injection of 6 mg/kg cDDP.

Solid tumours. Fig. 2 shows cDDP-DNA binding in RIF1 tumours 25 hr after ≥ 4 mg/kg cDDP. Nuclear staining was absent in tumour sections from untreated mice. cDDP-DNA binding was distributed homogeneously in all RIF1 tumours. Binding was also observed in the other tumour types (2, 24, and 48 hr after 10 mg/kg cDDP).

Cultured cells. Nuclear staining could be seen in HBE cells (Fig. 3), after exposure 10 µg/ml cDDP, or in RIF1 and CHO cells (Fig. 4). After treatment with 10 µg/ml cDDP, the ratio of staining intensity of RIF1 nuclei to the staining intensity of CHO nuclei was 8.5. Cell killing after treatment with cDDP (1 hr, 0-40 mg/kg cDDP) is shown in Fig. 5: RIF1 cells were much more sensitive towards cDDP than CHO cells.

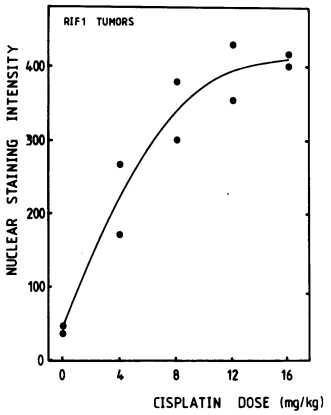

Fig. 2. cDDP-DNA binding in growing RIF1 tumours in C3H mice, 25 hr after an i.p. injection of 0-16 mg/kg cDDP.

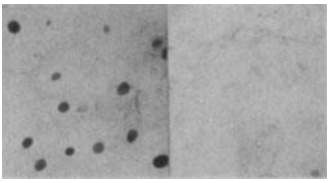

Fig. 3. cDDP-DNA binding in s.c. human buccal epithelial cells 1 hr after incubation with 0 µg/ml (right) or 10 µg/ml (left)

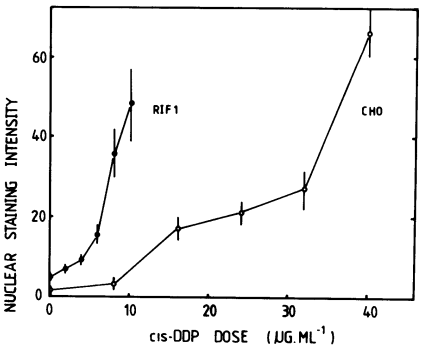

Fig. 4. cDDP-DNA binding in cultured tumour cells 3 hr after a 1 hr incubation with 0-40 µg/ml cDDP.

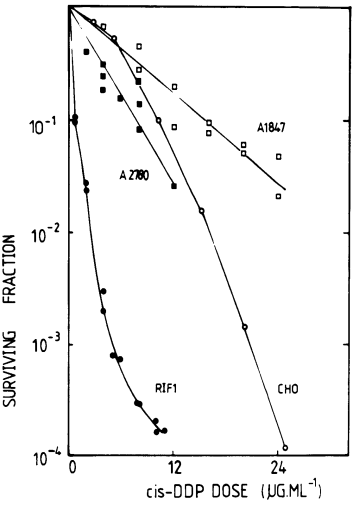

Fig. 5. cDDP-induced cell-killing of tumour cells in vitro (clonogenic assay).

DISCUSSION

The positive immunocytochemical results indicate that our antiserum is able to recognize in situ DNA with a low level of cDDP- or CBDCA-induced modifications. The results suggests epitopic similarity of at least a number of CBDCA- and cDDP-DNA interaction products (see also ref. 5). We also studied the

recognition of cDDP-modified DNA by the anti-cDDP-DNA antiserum in the competitive ELISA. The amount of Pt giving 50 % inhibition (IA_{50}) was 2-15 fmol Pt/100 µl when standard cDDP-DNA (rb: 9.10^{-2}) was used. However, IA_{50} values strongly increased with decreasing levels of cDDP-DNA binding. The immunocytochemical assay showed the persistence of cDDP-DNA interaction products in the kidney up to 162 days. This correlates quite well with the persistence of functional kidney damage in C3H/nu mice (6). Comparision of in vitro cultured RIF1 and CHO cells showed that sensitivity of these cells towards cDDP was inversely related to the cDDP dose needed to obtain a given level of initial nuclear staining. Preliminary results suggest that this correlation also holds true for human A2780 and A1847 tumour cells (results not shown). This would mean that, at least for the 4 cell lines studied, differences in cDDP-induced cell killing could be largely explained by different levels of cDDP-DNA binding as determined by our antiserum.

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QUALITATIVE INVESTIGATION, BOTH *IN VIVO* AND *IN VITRO*, OF THE METABOLITES FORMED BY CISPLATIN AND PARAPLATIN INVOLVING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

N.D. Tinker, H.L. Sharma and C.A. McAuliffe

INTRODUCTION

The two principle platinum based anti-tumour compounds in clinical usage at the present time are Cisplatin ($Pt(NH_3)_2Cl_2$; cis D.D.P.) and Paraplatin ($Pt(NH_3)_2(CBDCA)$).

Both drugs show activity against various types of cancers; however, the widespread usage of these compounds is hampered by severe side-effects; for cisplatin this takes the form of chronic nephrotoxicity, accompanied by nausea and vomiting, and myelosuppression for paraplatin.

The current belief is that the side-effects of these drugs may well be due to species formed by the 'metabolism' of these drugs, and that these metabolities may also contribute to the anti-tumour activity, since they often remain in the body longer than the parent compound.

It has been assumed that the metabolism of cisplatin, and hence other platinum compounds, involves simple chemical reactions rather than enzyme medicated reactions, since no enzymes are known that can act on platinum complexes and no evidence has been presented to establish enzyme type dynamics for cisplatin metabolism.

Cisplatin and the other platinum compounds which have been investigated are simple inorganic complexes and hence undergo ligand exchange reactions (1). In biological systems, the extent to which these reactions occur is limited to the availability and affinity of the substituting ligands. Of the commonly occuring ligands in biological fluids the order of affinity for Pt(II) is: sulphur > nitorgen > chloride > water, whereas the order in terms of concentration is the reverse (2). The aquation chemistry of cisplatin has been well studied (1); cisplatin forms a series of aquo species, where the chloride ligands have been

replaced by water, and since these aquo complexes are slightly acidic, they slowly dissociate to form mixed aquo-hyroxy and finally hydroxy complexes. It has been shown that these hydrolysis products are approximately 1000 times more reactive than the parent compound (3), and that the diaquo species, $(Pt(NH_3)_2(H_2O)_2]^{2+}$ is particularly toxic. Since these hydrolysis reactions are reversible, a high chloride ion concentration will inhibit hydrolysis; the chloride ion concentration in human plasma is approximately 103 mM and hence hydrolysis will be negligible, however, on passing into the cells of the body where the chloride ion concentration is about 4 mM, hydrolysis will presumably occur to a much greater extent.

However, though there has been much emphasis on aquation of cisplatin in the past, the presence of other potential ligands besides water should not be forgotten - plasma contains a wide variety of substances ranging from lipids, fatty acids, and urea to amino acids and proteins, many of which contain sulphur and nitrogen moieties capable of binding to platinum.

Studies of the metabolities of cisplatin, using chromatography have been carried out by several workers (4) and most recently by Daley-Yates and McBrien (5). The data presented in this paper attempts to continue these investigations on the platinum metabolites found in protein free plasma involving high performance liquid chromatography (HPLC). Our initial work was of a qualitative nature, though our later studies did attempt to study the relative concentrations of the different species formed in a semi-quantitative manner.

Since the platinum compounds to be studied were all water soluble to varying degrees, polar, and probably charged, the most suitable chromatographic conditions were those of reverse phase chromatography (RPC). RPC involves a hydrocarbon-bonded support (i.e. ODS which is a 18 carbon chain bonded onto silica) with an aqueous eluent containing a proportion of organic modifier. The mechanism of RPC involves the hydrophobic surface extracting the more lipophilic component of the eluent (i.e. the organic modifier) to form an organic rich layer at the particle surface in which the chromatographically useful partitioning takes place (6). The more polar solutes have greater affinities for the eluent and so elute in reversed order of polarity, that is, most polar first.

The system can be finely tuned by the use of ion-pairing agents. In our experiments, we used sodium dodecylsulphate (SDS). This is a water soluble salt, but due to the presence of the dodecyl carbon chain, it has the potential to form an ion-pair that is soluble in organic phases; due to the sulphate

moiety of the molecule, it will form strong ion-pairs which cations, e.g. Pt(II) aquo or hydroxy species. In theory, in the aqueous phase, the sodium counter ion is displaced by the positively charged Pt(II) species to form a polar organic molecule; this molecule then preferentially dissolves in the polar organic phase on the ODS surface. Thus the cationic Pt(II) ion is retained on the column - to what extent depends upon the concentration of the ion-pairing agent (the more agent, the greater the partition into the organic layer and the greater the retention time) and upon the amount of non-polar component in the eluent (the greater the covering of the ODS with organic phase the more lipophilic the column surface will be, causing longer retention times for the less polar components).

METHODS AND EQUIPMENT

The platinum compounds were prepared with Pt-195m as a radiolabel. Pt-195m ($t_{\frac{1}{2}}$ = 4.02 days) was produced by neutron irradiation of enriched Pt-194 (96.04%) in the Harwell reactor at Amersham International, Didcot, U.K., using a thermal neutron flux of 2.0 x 10^{14} neutrons $cm^{-2}s^{-1}$.

The platinum compounds synthesised have a high specific activity of 75 to 500 μCi/mg. This high specific activity, coupled with the fact that the Pt-195m is a gamma-ray emitter enables direct and sensitive detection of platinum at low concentrations.

The synthesis procedure for Pt-195m labelled compounds had been reported previously and was used with slight modifications in a few steps (7).

Compound purity was monitored using thin layer chromatography and high performance liquid chromatography.

The HPLC equipment consisted of two Shimadzu reciprocating pumps, a mixing chamber, a Rheodyne sample injector, and a Lichrosorb C_{18}ODS column (15 cm long).

Male Wistar rats of approximate weight = 150g, were used for the in vivo studies and the Amicon MPS-1 micropartition system, using YMT membranes with a 25k-30k Dalton cut-off, was used for the ultrafiltration of plasma.

The experimental procedure for the in vivo studies was as follows:
The platinum compound was dissolved in saline (0.9%) and immediately injected into the caudal vein of the rats. The dose was 15 mg/Kg, administered in 0.5 ml of solution. At selected time points, usually 0.5, 3 and 24 hours, the

animals were sacrificed and blood samples taken (about 5–6 ml) and placed in heparinised tubes. The blood was centrifuged at 3000 rpm for 10 minutes and the plasma removed. 500 μl of plasma was then placed in an Amicon ultrafiltration cone and centrifuged at 2000 rpm for 5 minutes. A 20 μl of plasma was immediately injected onto the HPLC column. Gradient elution conditions were used; eluant one consisting of 5 mM sodium dodecylsulphate (SDS) solution and eluant two of 70:30 acetonitrile:water. The gradient was 100% eluant one to 100% eluant two in steps of 42.75 seconds duration, the gradient being controlled by a microcomputer system. The flow rate was 800 μl/min. The eluant was collected using a Pharmacia Frac–100 fraction collector, with a fraction size of 0.60 mins. (0.48 ml). Total run time was 57 minutes, with 94 fractions. The resulting fractions were then counted using a LKB–Wallac Ultragamma counter – with an energy window of 50 – 174 KeV (enabling the two most intense energies of Pt–195m, 98.8 and 129.7 KeV, to be monitored). The counter automatically corrected for background and isotopic decay during counting.

The in vitro experimental procedure was as follows:
The compounds were incubated in a minimum amount of saline or water and brought to 37°C, similarly a sample of plasma. At equilibrium, the compound was added to the plasma (dilution being kept to a minimum) and incubated at 37°C in the dark. Samples were removed at fixed time intervals and centrifuged down using ultrafiltration cones, and a sample of the protein free plasma injected onto the column.

RESULTS

A) Cisplatin metabolism

Initial studies were carried out involving cisplatin incubated in saline and water. In 0.9% saline solution there was no change in cisplatin peak area over 48 hours, confirming the expected stability of cisplatin in solutions of high chloride concentration. Incubation of cisplatin in water (1 mg/ml) showed a broad peak (B) (fraction 9–20) eluting soon after cisplatin (A) (fraction 4) and another peak (C) (fraction 56–60) appearing after only 1 hours incubation, which remained much longer on the column. Fig.(1).

The broad peak is probably several unresolved species, e.g., hydroxy and aquo species $[Pt(NH_3)_2(OH_2)Cl]]^+$, $[Pt(NH_3)_2(OH)_2]$. Peak C is eluting after

about 34 minutes, retention volume 28 mls, and this corresponds to the elution time of a prepared sample of $[Pt(NH_3)_2(H_2O)_2]^{2+}$, under these conditions. The peak is broad due to the length of time on the column. The diaquo species is expected to remain on the column for a considerable length of time since it forms a strong ion-pair with SDS, being a dication.

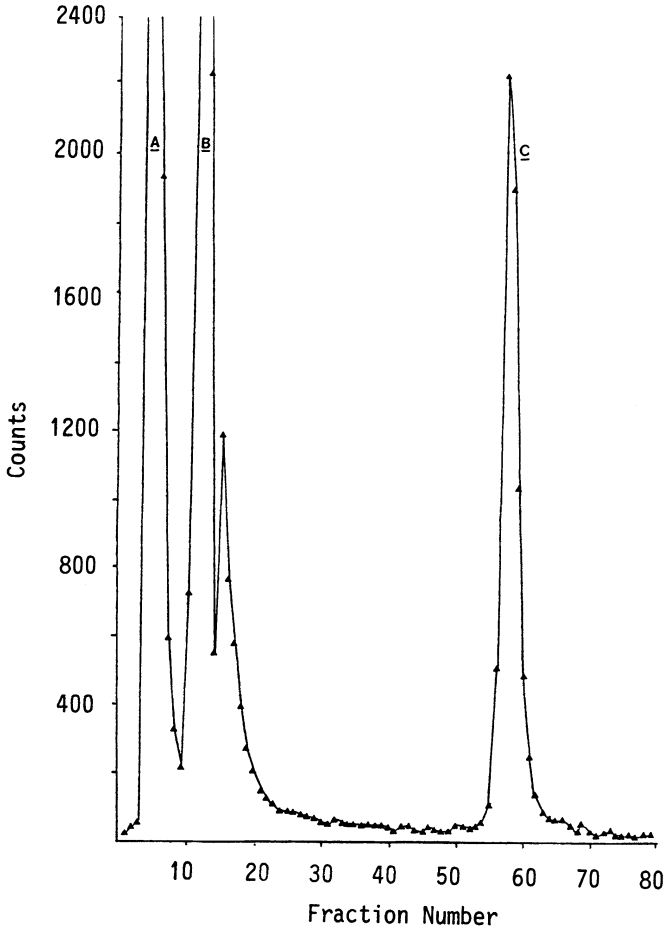

Fig.1. The HPLC elution profile of cisplatin after incubation for 1 hour in water (1 mg/ml). Flow rate = 0.8 ml/min. and fraction size = 0.6 minutes. A is cisplatin.

A sample of cisplatin in water was incubated for 47 hours, after which time sodium chloride was added and the mixture incubated for a further 1 hour. Fig.(2) shows that peak (B) has totally disappeared - this would suggest that this peak consists of simple aquo, aquo-hydroxy and/or aquo-chloro species which are

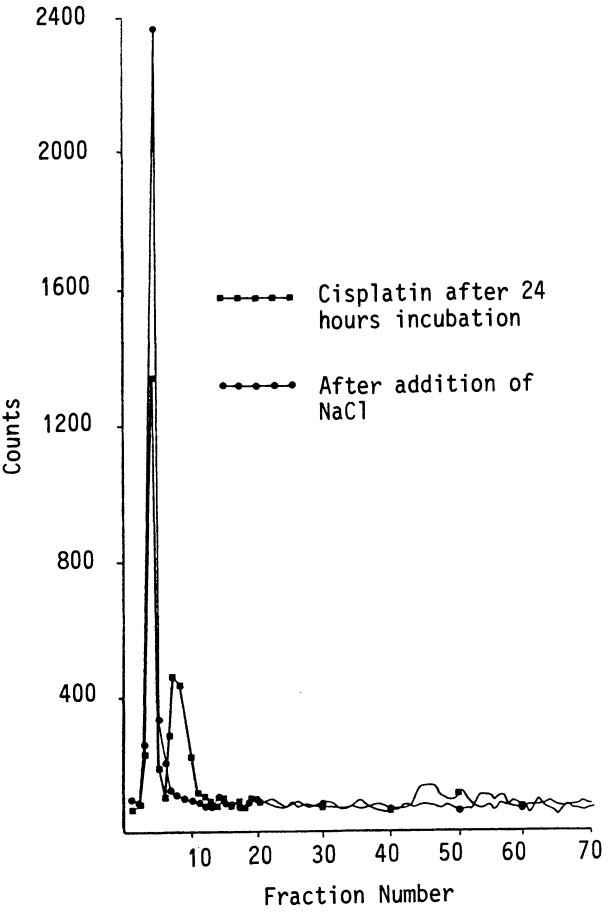

Fig.2. The elution profile of cisplatin (A) incubated for 47 hours in water and after a further 1 hours incubation with NaCl.

converted back to cisplatin due to the high chloride concentration. Di-hydroxy and bridged hydroxy species, which would form via hydrolysis, are unlikely to undergo ligand exchange reactions with chloride ions.

i) Cisplatin in-vivo

For this experiment, rather than dosing three animals (per time point) at the same time, three series' of injections were carried out on consecutive days and the data averaged for each time point. Table (1). The relative concentrations of platinum containing species were monitored by integrating peak area and expressing this as a percentage of total peak areas and following this with time.

Two principle peaks were observed at each time point, the cisplatin peak and peak (C). A plot of percentage peak area versus time is shown in Fig. (3). The levels of cisplatin were found to decrease exponentially with time (taking cisplatin to be 100% at 0 hours) within the first three hours, but after this period equilibrium appears to be reached. There was no evidence of aquo species in the plasma from any of the rats. For the 24 hour time points, the levels of platinum were approaching the detection limit of the experiment.

It is clear that cisplatin is rapidly metabolised despite the high chloride concentration in plasma; the number of metabolites observed is less than reported in previous studies (5,8) - although in principle it may be that between the 0.5 and 3 hour time points several intermediate species may have formed and then further reacted.

ii) Cisplatin in-vitro

Fig. (4) shows the presence of cisplatin and the principle metabolite (C) in rat protein free plasma 24 hours after incubation of plasma at 37°C with cisplatin (1.2 mg/ml). The cisplatin and metabolite (C) are eluting at the same position as in the in vivo experiment, which is as expected if metabolism is by chemical rather than biochemical pathways. Samples were eluted at 0.5, 3, 5, 24,and 48 hours; small peaks are observed thoughout the experiment but they never exceed 2-3% of the cisplatin concentration. Metabolism appears slower than in previous studies, with 67% of the activity due to cisplatin even after 48 hours - this may be due to chloride contamination, which partially inhibits

TABLE 1 CISPLATIN METABOLITES FORMED IN RAT PLASMA *IN VIVO*

TIME / Hours	ANIMAL	TOTAL COUNTS / Min	PEAK COUNTS Fraction A / Min	PEAK COUNTS Fraction C	Peak counts of A expressed as a %age of total peak counts	Peak counts of C expressed as a %age of total peak counts	AVERAGE %age PEAK A ± S.D.	AVERAGE %age PEAK C ± S.D.
0.5	R2	13,362	9,749	1,421	73.0	10.6	79.0 ± 7.2	8.3 ± 2.5
	R6	34,324	26,470	2,925	77.1	8.5		
	R8	28,022	24,441	1,608	87.0			
3	R3	12,179	2,264	3,595	18.6	29.5	19.6 ± 5.2	26.4 ± 3.6
	R5	6,335	1,602	1,727	25.3	27.3		
	R9	10,334	1,551	2,329	15.0	22.5		
24	R1	6,195	1,717	1,956	27.7	31.6	23.4+ ± 6.1	29.8+ ± 2.6
	R4	7,090	1,351	1,976	19.1	27.9		
	* R10	-	-	-	-	-		

* Pt levels below detection

\+ Mean of two animals

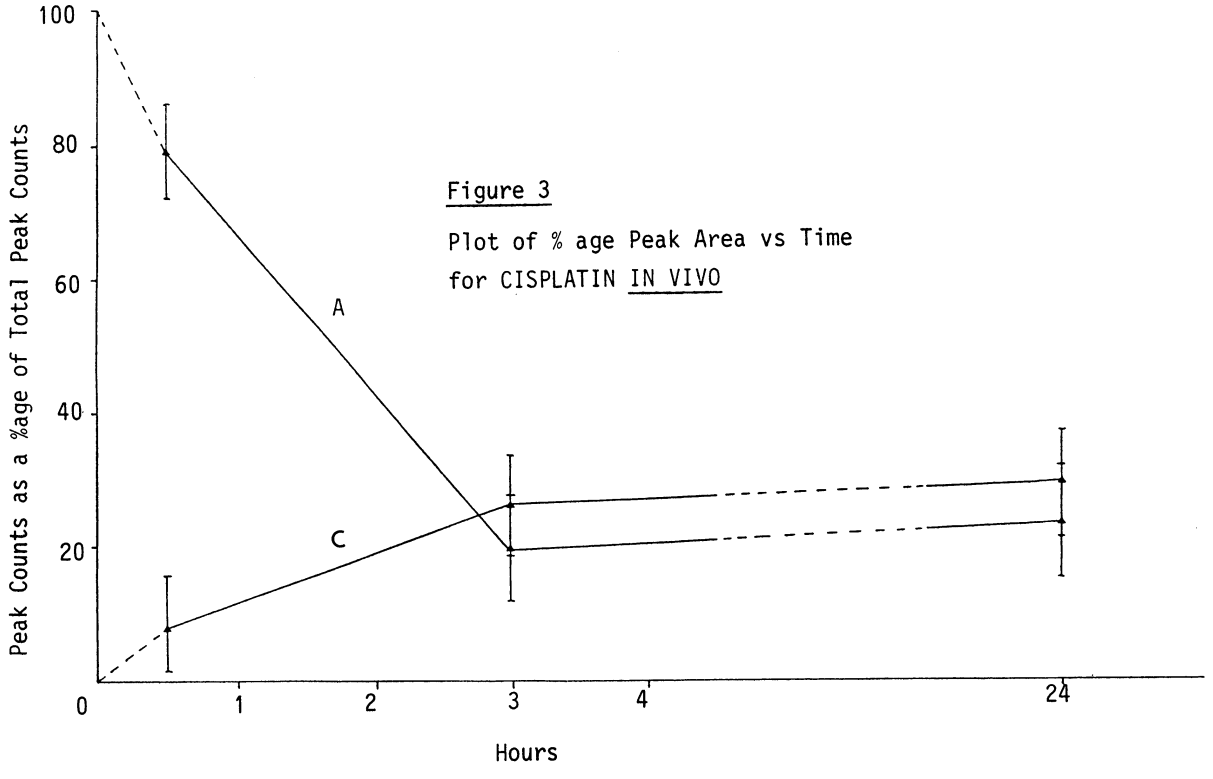

Figure 3

Plot of % age Peak Area vs Time for CISPLATIN IN VIVO

hydrolysis. Peaks which Daley-Yates and McBrien observed and attributed to the platinum-methionine complexes (8) were not observed in our experiments.

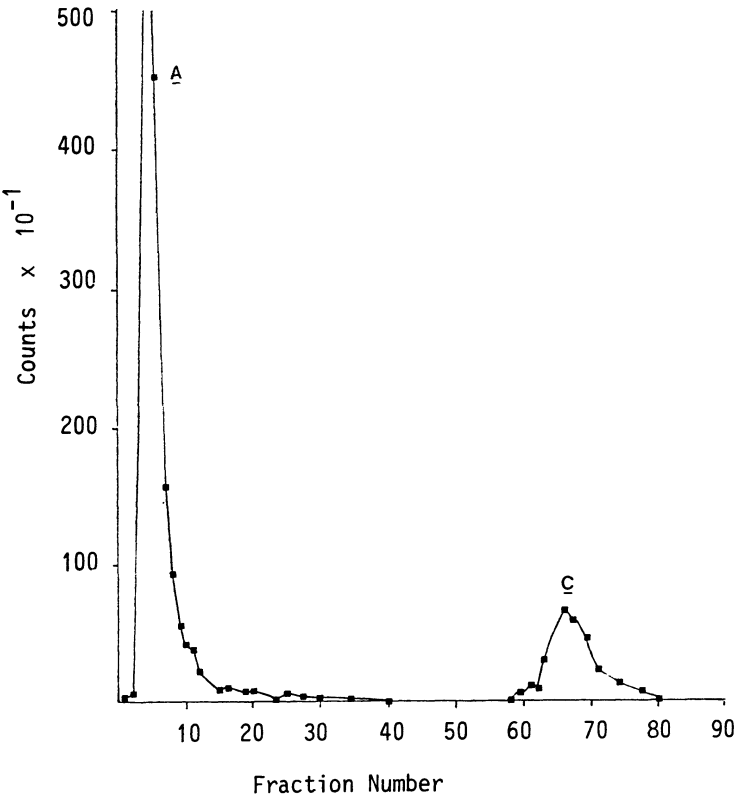

Fig.4. The elution profile of cisplatin incubated in rat plasma for 24 hours. A is cisplatin; maximum of peak is at 6750 counts.

B) Paraplatin metabolism

Preliminary studies were carried out regarding the stability of paraplatin in both water and saline media. The concentration of paraplatin incubated in water (19.5 mg/ml) was monitored over a 43 hour period. There was negligible change in concentration (<1%) over this period, indicating paraplatin is stable to hydrolysis within this time limit. Fig. (5) shows the elution profile of paraplatin

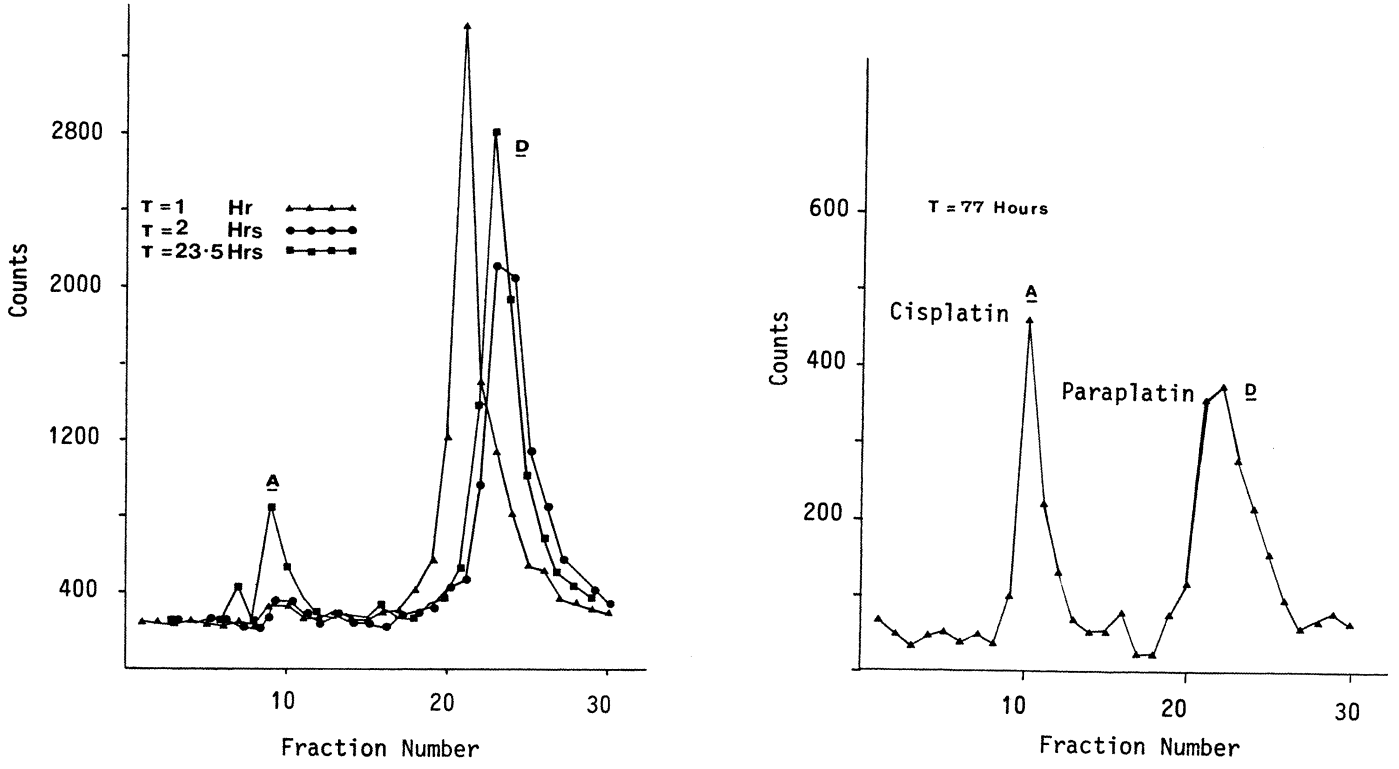

Fig.5. The elution profile of paraplatin in 0.9% saline solution (1.5 mg/ml) over 77 hours. A - cisplatin; D - paraplatin.

incubated in 0.9% saline (1.5 mg/ml) over a 77 hour period. Paraplatin has an elution volume of about 3.6 mls (fraction 22.5) under these experimental conditions; cisplatin under the same conditions elutes at 1.6 mls (fraction 10).

From Fig. (5) it can be seen that the paraplatin concentration decreases and a peak which behaves chromatographically like cisplatin forms after 1–2 hours and increase in magnitude – this clearly indicates that paraplatin is converted to cisplatin (this has been suggested by previous workers (9)); no other peaks are observed, presumably since the cisplatin cannot further hydrolyse due to the high chloride concentration.

An in vivo study of the metabolism of paraplatin was carried out using the procedure as outlined above for cisplatin.

Fig. (6) illustrates the average elution profiles for the 0.5 and 3 hour time points. Clearly paraplatin (fracation 9–10) is being rapidly converted to cisplatin (fraction 4) in vivo, with the presence of further metabolities evident, even after 0.5 hours, at fraction 55–80. By the 24 hour time point levels of platinum in the protein free plasma had fallen below detection limits in all three animals.

Equivalent results are obtained in the in vitro experiment. 6 mg of drug was incubated with 1 ml of rat plasma; samples were taken at 0.5, 1.5, 3, and 5 hours. All three peaks are observed, Fig (7); cisplatin at fraction 4, paraplatin at fraction 7–10, and the other metabolite(s) at fraction 55–70. However, the relative concentrations of the three 'peaks' is different, even allowing for the semi–qualitative nature of the experiments; the conversion of paraplatin into cisplatin is much slower in vitro than in vivo, and the relative concentration of the broad peak eluting at fraction 55–60 is much lower in vitro than in vivo. It is unclear whether or not this is simply a concentration effect.

The fraction 55–60 peak could be a single species, for example the diamminediaquo platinum (II) species, its broad nature being due to the length of time on the column, though this requires further investigation.

In summary, it can be said that both in vivo and in vitro paraplatin reacts to form cisplatin, involving the displacement of the cylobutane – dicarboxylato ligand by chloride ions and that the cisplatin further metabolies.

A similar set of experiments with iproplatin (CHIP) are in progress.

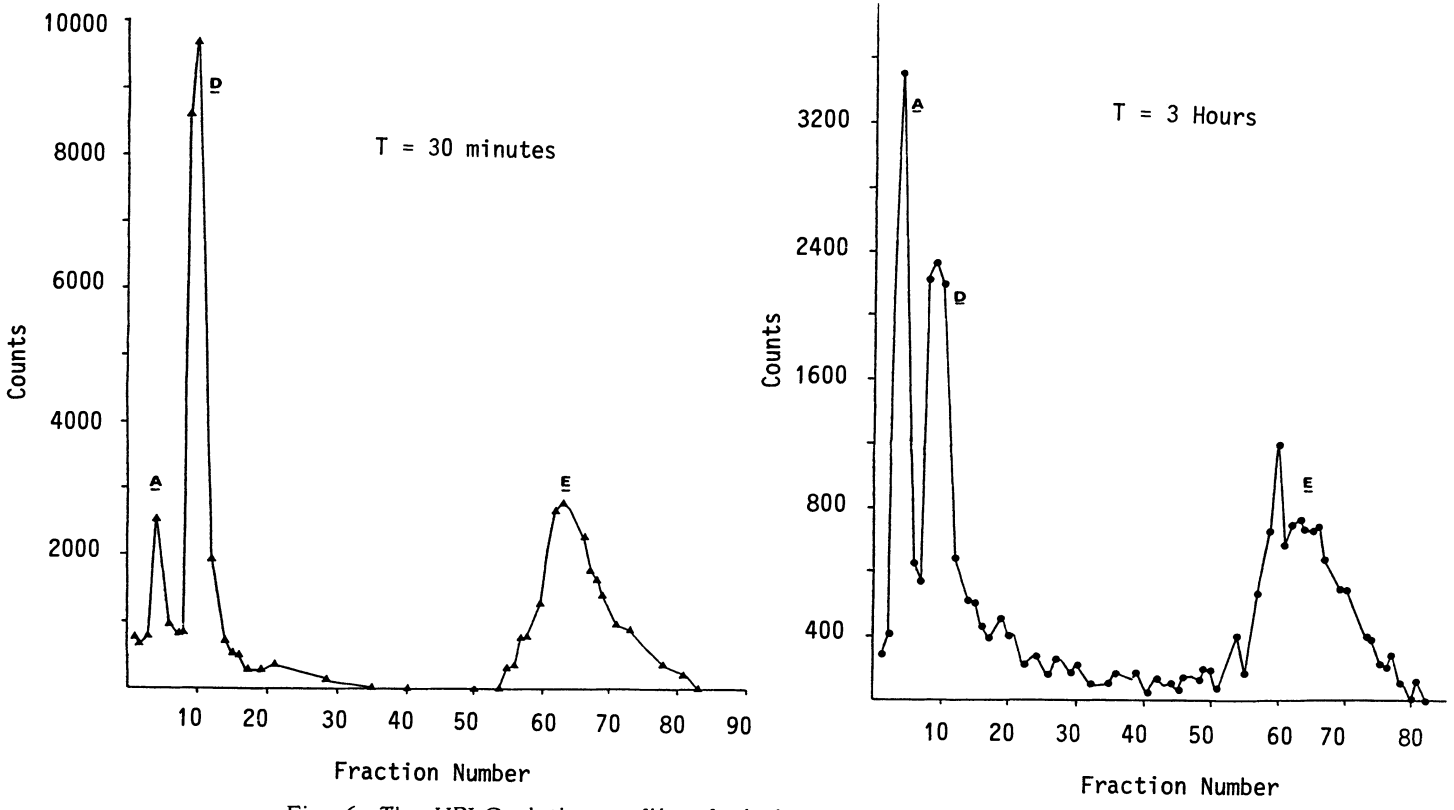

Fig. 6. The HPLC elution profile of platinum species in rat protein free plasma 30 minutes and 3 hours after dosing with paraplatin (15 mg/Kg). D is paraplatin.

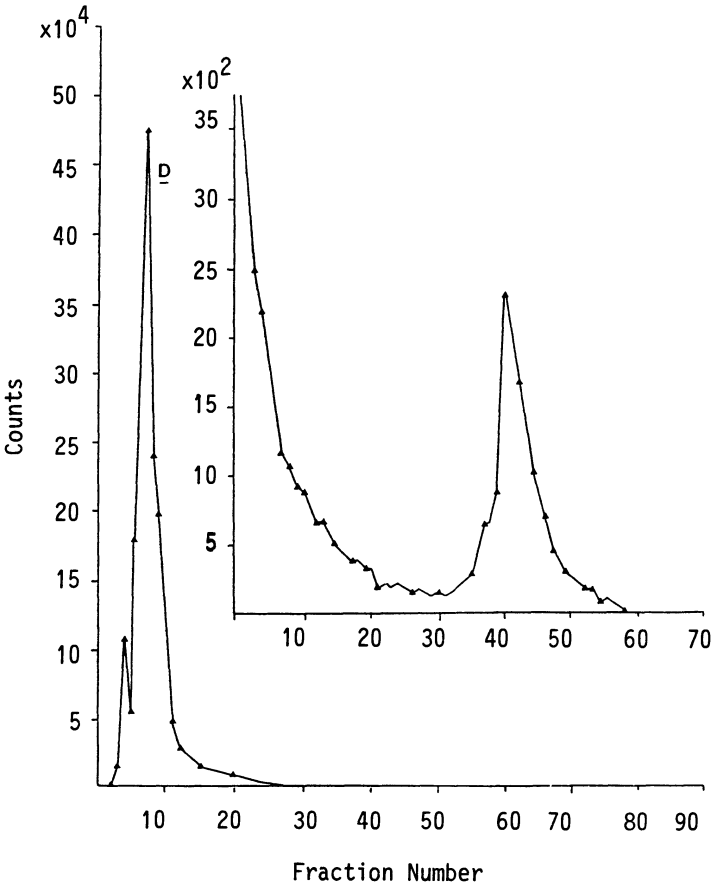

Fig.7. The elution profile of paraplatin incubated in rat plasma over 5 hours. D - paraplatin.

DISCUSSION AND SUMMARY

Both the in vitro and in vivo studies involving cisplatin show that cisplatin is rapidly metabolised in such systems, despite the high chloride ion concentration intrinsic in those systems. The main peak detected other than that due to the parent compound is a broad peak eluting at fraction 56–60, which may well be due to several unresolved hydroxy and aquo species. Another

peak, B, eluting soon after cisplatin is probably due to simple aquo species as it is chloride ion concentration dependent. This peak is not observed in the in vivo study, although it may be that between the time points used, the relevant species form and further react.

Studies with paraplatin clearly show the conversion of this compound to cisplatin in both in vitro and in vivo systems, and that the cisplatin probably further metabolises.

The evidence strongly indicates that the metabolic peak C of cisplatin is due in part at least to the diamminediaquoplatinum (II) complex. This is known to be toxic and several workers have aluded to this species as a potential nephrotoxic agent, (8).

Paraplatin may exert its anti-tumour activity via its conversion into cisplatin, though this does not explain its lack of nephrotoxicity. When paraplatin is used therapeutically approximately 5 times as much platinum is given than when cisplatin is used (9). Hence the amount of cisplatin formed via paraplatin conversion may be similar to a normal therapeutic dose, however, this would mean that the cisplatin nephrotoxic agent would also be formed to approximately the normal extent. The rate at which the cisplatin forms will obviously have an effect on the levels of agent and toxic species found in the plasma at any one time, but the lack of nephrotoxicity of paraplatin strongly suggests the occurance of other reactions besides conversion into cisplatin, since cisplatin nephrotoxicity is accumulative and occurs at very low doses.

ACKNOWLEDGEMENTS

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B: Pharmacological Mechanisms and Toxicology of Platinum Complexes as Antitumor Agents.

CISPLATIN EFFICACY AND TOXICITY: ARE THEY SEPARABLE ?

M.P. Hacker and J.D. Roberts

INTRODUCTION

Rosenberg first reported the antitumor activity of cis-diamminedichloroplatinum (II) (DDP) in 1969 and since that time DDP has become one of the most widely used cancer chemotherapeutic drugs in the clinic. DDP has marked activity in several types of tumors including testicular, ovarian and head and neck carcinomas (1-3). In spite of the impressive activity of DDP in selected tumors a number of factors, such as a limited spectrum of responsive tumors, severe host toxicity, the development of resistance by tumor cells and relatively poor water solubility, have hampered the clinical usefulness of this drug.

Toxicities, and especially non-myeloid toxicity, associated with the clinical use of DDP have presented the greatest number of problems for the patient. Such adverse effects as nausea, vomiting, renal tubular damage, ototoxicity, peripheral neuropathy, myelosuppression, tetany and allergic responses are observed in patients treated with DDP (4). Obviously, the elimination or even significant diminution of one or more toxicities without a concomitant decrease in oncolytic activity would greatly enhance the utility of DDP. To this end, a great deal of effort has been put forth in an attempt to increase the therapeutic ratio of DDP. The purpose of this report is to provide an overview of a number of experimental and clinical investigations taken to achieve this goal. In an attempt to provide focus to this endeavor, the author has chosen to

concentrate on the nephrotoxicity, nausea/vomiting, and ototoxicity that accompanies DDP administration.

DDP-INDUCED NEPHROTOXICITY

The nephrotoxic potential of DDP was first recognized in the preclinical toxicological testing and has been demonstrated in 20-30% of patients receiving single dosages of 50 mg/m^2 of body surface area (5). In early clinical trials, it soon became obvious that while protracted nausea and vomiting were frequently the most distressing symptoms for the patient, nephrotoxicity was usually the dose-limiting toxicity. Fortunately, nephrotoxicity in many cases is mild and reversible. However, since the toxicity has been demonstrated to be both dose dependent and cumulative, a life threatening, irreversible damage to the kidney can result at higher DDP dosages.

Microscopic examination of renal tissue following DDP administration reveals focal necrosis of the distal convoluted tubules and collecting ducts, dilation of the convoluted tubules and cast formation (6). The onset of DDP-induced nephrotoxicity usually occurs during the second week after initiation to therapy and is manifested by a rise in BUN and/or serum creatinine values with a corresponding decline in creatinine clearance (7).

It should be stated here that which, if any, of the standard clinical chemistry values is the best indicator of kidney damage is currently a point of debate. In a recent study Mejier *et al.* demonstrated a significant fall in glomerular filtration rate (GFR) without a corresponding rise in serum creatinine or β_2-microglobulin concentrations (8). Further, Safirstein *et al.* have reported that polyuria and reduced GFR persist long after BUN values have returned to normal levels (9). Finally, Daugaard *et al.* have shown similar results using high dose DDP and concluded that DDP causes both a tubular and glomerular defect in renal function (10). Taken in aggregate, these results suggest that it is extremely important to identify which assay or battery of assays best indicates developing kidney damage before we can

positively state whether a given renal protective maneuver is truly effective.

With that caveat, the discussion will now concentrate on experimental and clinical approaches taken to minimize DDP nephrotoxicity. Numerous strategies have been developed thus far and include osmotic diuresis in combination with hydration, hypertonic saline, the use of sulfur nucleophiles and the development of non-nephrotoxic DDP analogs.

It has been shown that vigorous hydration in combination with osmotic diuretics such as mannitol can significantly decrease the incidence of DDP nephrotoxicity without interfering with the antitumor activity of the drug (11). While the mechanism of this intervention is not completely understood, it has been proposed that hydration and diuresis may decrease the urinary concentration of platinum and perhaps decrease the exposure of the renal tubules to free platinum species. Although somewhat effective, a sufficient number of patients receiving this therapy still developed DDP-induced nephrotoxicity to warrant further investigations.

More recently, Litterst demonstrated that DDP nephrotoxicity in mice was altered by the vehicle in which DDP was administered (12). Whereas DDP prepared in water and administered at 15 mg/kg killed 50% of mice, no toxic deaths were observed when DDP was dissolved in a 4.5% NaCl solution and administered to mice (Table 1).

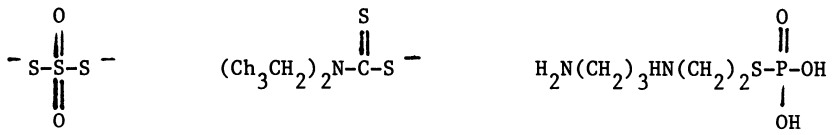
Table 1

NaCl Concentration (g/100 ml)	Percent Mortality ($n \geq 10$)
0.45	100
0.9	80
1.8	37
4.5	18

In a series of studies Ozols et al. have clearly demonstrated that a similar approach can be used clinically (13, 14). No significant change in creatinine clearance or serum creatinine levels was observed in patients receiving DDP at a dose of 40 mg/m^2 when dissolved in 250 ml of 3% NaCl daily for 5 days even after 3 or 4 courses of this therapy. These patients also received vigorous hydration and diuresis when necessary with each dose, which may have helped diminish DDP nephrotoxicity.

The success of this approach has led Ozols to conclude that nephrotoxicity is no longer a clinical problem. This conclusion is not universally accepted, however, as several investigators have reported a disturbing incidence of nephrotoxicity in spite of employing hypertonic saline.

Currently 3 sulfur-containing chemoprotectors are undergoing extensive preclinical and/or clinical evaluations (Figure 1).



TS DDTC WR-2721

Fig. 1

All 3 compounds, diethyldithiocarbamate (DDTC); thiosulfate (TS); and WR-2721 appear to act by binding through the S moiety to the platinum, thereby inactivating DDP. These agents are all anionic, hydrophilic and can be administered under conditions where nephrotoxicity is diminished without an apparent reduction in antitumor effect. It is important to note that for each agent the schedule of administration is critical; as some rescue schedules can result in a loss of nephroprotectant activity, negation of antitumor activity of DDP, or both.

Why these chemoprotectants are apparently selective is only somewhat understood. DDTC appears to act as a true rescue agent being able to: 1.) remove platinum from the kidney; 2) restore

the activity of platinum inhibited enzymes; and 3) prevent systemic platinum toxicities other than the kidney (15). DDTC does not remove platinum from bis guanine complexes in DNA, which could explain the selectivity of DDTC, especially if nephrotoxicity is not related to platinum-DNA interactions whereas cytotoxicity is. While clinical experience is limited with DDTC, there is suggestive evidence that DDTC may well have nephroprotective potential. A concern that has arisen in these clinical studies is the disturbing toxicity observed upon DDTC administration, in which patients experience transient burning of the mouth, chest tightness, and extreme anxiety. Whether this toxicity can be ameliorated by altered administration protocols or concomitant drug therapy remains to be determined (personal communication).

TS has a very narrow administration window in that it must be administered just prior to or concurrent with DDP (Table 2).

Table 2

Time Interval Relative to DDP (Hr)	BUN (mg/dl)
-4	105
-1	45
0	25
+1	85
+4	100

TS appears to have no effect on DDP toxicities other than nephrotoxicity nor does TS regenerate platinum inhibited enzymes (16). Presently, it is hypothesized that TS nephroprotection may result from concentration of TS in the renal proximal convoluted tubules and <u>in situ</u> inactivation of DDP. To date the most impressive clinical effects of TS have been with intraperitoneal DDP administration in the treatment of ovarian carcinoma. Using

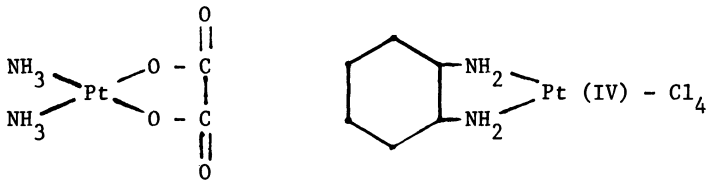
this route of DDP administration with concurrent systemic TS administration as much as 270 mg DDP/m^2 can be given without causing a significant rise in serum creatinine (17).

WR-2721 selectivity is least well understood. It is known that WR-2721 must undergo hydrolysis at the thiophosphate ester to generate the aminothiol which, in principal, could form an inactive tridentate chelate with DDP (18). Yuhas had proposed that WR-2721 is preferentially taken up by normal cells which may explain its selectivity for protection. WR-2721 is only effective when administered just prior to DDP. While WR-2721 has little inherent toxicity and can have remarkable activity in protecting against the nephrotoxicity of DDP, it appears as though there is a maximum tolerated dose for DDP even when WR-2721 is used, and that this maximum dose may be less than that achievable with either DDTC or DS.

Amelioration of nephrotoxicity has led to the clinical use of higher doses of DDP in which other toxicities are now dose limiting. The reason for the development of additional dose limiting toxicities is a function, in part of the relative selectivity of the nephroprotectant used. In high dose DDP protocols utilizing hypertonic saline or TS, neurotoxicity has been dose limiting (19). This toxicity, resulting from loss of myelinated nerve fibers, appears to be related to total dose administered and only partially reversible.

At a recent symposium Oriana *et al*. reported some very exciting early clinical results in which patients were treated with reduced glutathione in combination with high dose DDP (20). In these studies, neither nephrotoxicity nor neurotoxicity was observed even at doses of DDP that routinely cause neurotoxicity in other high dose DDP protocols. In addition, no decrease in expected oncolytic activity was noted suggesting that this was a selective protection. These observations await further large scale studies to document the ultimate success of this intriguing report.

In addition to seeking interventions that will modify the nephrotoxicity of DDP, considerable effort has been devoted to the development of other platinum analogs that retain the antitumor properties of DDP but are considerably less toxic. To date several platinum based complexes have entered clinical trials. Of these, only carboplatin (CBDCA or [1,1-cyclobutane dicarboxylato-(2)-0,0']-diammino platinum) and tetraplatin (1,2-diaminocyclohexane)-tetrachloro platinum (IV) (see Figure 2) appear to have sufficiently favorable attributes to warrant large scale clinical trials.



CBDCA Tetraplatin

Fig. 2

CBDCA appears to be significantly less toxic and less emetogenic (21). One problem with CBDCA is the fact that it has stable amine ligand identical to DDP and appears to be cross resistant with DDP in tumor cells. Tetraplatin is currently undergoing Phase I studies in Europe and should enter Phase I studies in the U.S. this year. This complex appears to be relatively non-nephrotoxic and because of the differing stable amine ligand could be non-cross-resistant with DDP (22). It is too early to tell whether this complex will advance to Phase II or III studies.

In summary, great strides have been made towards eliminating, or at least diminishing, the nephrotoxicity of DDP. Whether this toxicity, as some believe, has become a nonentity in the clinical setting needs further substantiation. One can safely state, however, that DDP-induced nephrotoxicity can be effectively diminished without concomitant loss of antitumor activity.

DDP INDUCED NAUSEA AND VOMITING

Patients receiving cancer chemotherapy often experience nausea and vomiting. Indeed, DDP has been referred to as one of the most nauseating anticancer drugs ever administered to humans. The inability to control this side effect of DDP can prevent successful treatment even in patients with a favorable prognosis. The potential to compromise the beneficial effects of DDP has lead to an intensive search for means of ameliorating this toxicity. Two areas of research, i.e. the use of antiemetics and the development of less emetogenic platinum analogs, have received the greatest attention.

In order to discuss this response to DDP properly a brief overview of the physiology of emesis is required. The vomiting control mechanism consists of two topographically distinct and functionally different units of the medulla oblongata, the emetic center (EC) and the chemotactic trigger zone (CTZ) (Figure 3).

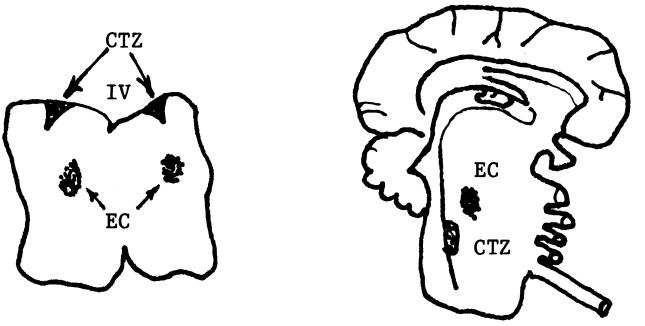


Fig. 3

The CTZ is located in the floor of the fourth ventricle and is accessible from the blood and the cerebrospinal fluid. Ablation of the CTZ causes an animal to become refractory to a variety of blood borne drugs and toxins. In contrast, there is no known emetic agent that acts directly on the EC. Rather, the EC, which is the final common pathway that mediates all vomiting, is stimulated by the CTZ and emesis occurs. Another potential input into the EC is the cerebral cortex, which may explain why many patients vomit with only the anticipation of receiving chemotherapy. Finally, there is a peripheral afferent nerve input

originating from the gastrointestinal tract that can cause emesis through stimulation of the EC.

In spite of the fact that nausea and vomiting are quite common, surprisingly little work had been done to elucidate the site of emetic action of chemotherapeutic agents until recently. Although the data are limited, it appears that different classes of chemotherapeutic agents initiate vomiting at different sites and that a single cytotoxic drug can trigger emesis simultaneously at more than one of the three areas known to be able to induce emesis. DDP, which has poor penetration of the blood brain barrier and a delayed onset of action, apparently causes emesis through stimulation of the visceral afferent nerves and the CTZ (23).

A major impediment to research in this area has been the choice of the appropriate animal model. Most small species of animals including the mouse, rat, guinea pig and rabbit do not vomit. While both the dog and cat are useful for such studies, they are expensive and because of their size require relatively large quantities of drug to perform the study. Recently, Schurig et al. reported that the ferret was an excellent model for studying the emetic effects of anticancer drugs and could be used as a means for studying the potential of antiemetic drug (24).

Several single agents have been identified for the control of cancer chemotherapy induced nausea and vomiting. Drugs evaluated thus far and found active include antihistamines, dopamine antagonists, cannabanoids and corticosteroids.

Further therapeutic benefit has been observed when active agents are used in combination. When antiemetics are combined with Lorazepam patient tolerance to both chemotherapy and antiemetic therapy is greatly enhanced. With these interventions the majority of patients do not vomit at all during the first 24 hr following DDP treatment (25).

Of the many antiemetics tested to date, metoclopramide, a benzamide with both peripheral and central antiemetic actions, has met with the greatest success as a single agent. The mechanism of action is most probably via a direct effect on the CTZ by blocking

dopamine receptors. However, one cannot rule out the peripheral effects of metoclopramide on the gastrointestinal smooth muscle via a cholinergic mechanism. In most cases metoclopramide is administered in relatively high dosages, especially when used as a single agent, and can lead to significant toxicities of its own such as lassitude, anxiety, agitation or restlessness, diarrhea or extrapyramidal reactions (26). In attempts to lower metoclopramide dosages, other drugs such as dexamethasone or diphenhydramine have been used with good success in combination with metoclopramide (27).

Despite this control of early vomiting most patients do experience nausea and vomiting 24 or more hours after drug administration. In a recent report, Kris et al. reported that the vast majority of patients experienced some degree of nausea or vomiting from 24 to 120 hrs after DDP (28). The reason for the relative lack of success in controlling delayed nausea and vomiting is an area needing further study.

An alternative approach to the use of antiemetics in combination with DDP has been the development of less emetogenic analogs of platinum. Using beagle dogs, Hacker et al. reported that CBDCA caused far less emesis than did DDP or a variety of other platinum analogs (29). These studies have since been substantiated by Schurig et al. in the ferret (24). More recently clinical studies suggest that while CBDCA is emetogenic in humans it is far less severe than DDP.

As with the nephrotoxicity it appears as though it is possible to adequately protect the patient from much of the nausea and vomiting associated with DDP treatment. Further, it appears as though through proper manipulation of the platinum complex it may well be possible to develop active complexes with significantly decreased emetic potential. This latter approach may be most important if control of delayed DDP nausea and vomiting continues to be a disappointment. Obviously, more work is required in this area.

DDP INDUCED OTOTOXICITY

The incidence of DDP ototoxicity differs markedly from study to study ranging from 0 to 90%. One of the reasons for this large discrepancy is the method whereby the investigators evaluated ototoxicity. For the most part a low incidence of ototoxicity is reported in investigations relying primarily on subjective hearing loss in the patient. Conversely, a high incidence of ototoxicity is reported in investigations employing audiometric tests. Using the latter approach, ototoxicity is usually manifested by high frequency hearing loss in the greater than 2000 Hz range. However, these same studies report a 10-15% incidence of hearing loss in the normal speech range (250-2000 Hz) (30).

Regardless of the incidence of ototoxicity reported, most authors agree that this toxicity is directly related to the dose of DDP administered. Other factors that appear to predispose a patient to ototoxicity include increased age, abnormal pretreatment audiograms, and pretreatment with aminoglycosides.

The pathophysiology of ototoxicity is not completely understood. It is well established that the primary lesion is cochlear damage resulting in loss of outer hair cells in the basal turn of the cochlea. Electron microscopy revealed progressive damage to the outer hair cell membrane and increased numbers of lysosomal bodies in the cytoplasm. This histologic picture is quite similar to the one seen in aminoglycoside cochlear damage.

While no biochemical mechanism of ototoxicity has been substantiated, it has been postulated that the outer hairy cell, which has a higher metabolic rate than the inner hairy cells, may be more susceptible to DDP effects on cellular metabolism. Guarino *et al.* reported that DDP inhibits Na^+-K^+-ATP'ase in isolated rat kidney slices (31). If DDP inhibits this enzyme in the inner ear, an accumulation of Na^+ in the endolymph can occur and edema develop. The edematous process has been hypothesized as the reason for the cellular destruction and subsequent auditory impairment.

Attempts to minimize this toxicity have included altered treatment schedules, use of chemoprotectants and the development of new platinum coordination complexes. While the majority of these studies have been done in the clinic, there has been a recent increase in the number of experimental preclinical studies. The primary model for DDP-induced ototoxicity is the guinea pig but the rat appears to have histologic changes in the inner ear similar to humans.

The treatment schedule may play a significant role in this toxicity. Approximately 30% of patients receiving 5 daily bolus injections of DDP had a measurable decrease in hearing acuity whereas only one of 96 patients had hearing loss when the same total amount of DDP was administered as an infusion. Vermoken et al. also reported a significant decrease in the incidence and severity of ototoxicity when DDP was administered as an infusion rather than a bolus (32). These studies suggest that, as with the aminoglycosides, DDP ototoxicity is related to peak serum levels.

To date no clinical intervention such as hydration and diuresis or hypertonic saline has diminished this toxicity. In fact the high dose DDP that can now be administered as a result of the nephroprotection afforded by hypertonic saline has resulted in a large number of patients having high frequency hearing loss.

CBDCA appears to be less ototoxic than DDP. Significantly less histopathologic damage was observed in guinea pigs treated with equitoxic levels of CBDCA compared with DDP. Further, preliminary clinical studies using CBDCA indicate that this analog of DDP has less ototoxic potential than the parent complex. These results are encouraging and suggest that the development of second generation platinum complexes with altered ototoxic potential without decreasing the antitumor activity may well be possible.

Schweitzer et al. recently published results showing that fosfomycin, a phosphonic acid antibiotic, significantly decreased DDP-induced ototoxicity in guinea pigs (33). In this study guinea pigs were treated daily with DDP (1 mg/kg/day subcutaneous) with or without fosfomycin (320 mg/kg/day subcutaneous). Whereas a 30 to 60% total loss of hearing occurred in guinea pigs receiving

DDP-alone, only a rare very mild loss of high frequency hearing was detected in guinea pigs receiving DDP + fosfomycin. Further, fosfomycin appeared to have marked nephroprotective properties. Unfortunately, no protection of either gastrointestinal enteropathy or peripheral neurotoxicity was observed. If these results are substantiated in other species and if fosfomycin does not decrease the antitumor activity of DDP, this chemoprotective agent could prove to be a valuable addition to the clinical use of DDP.

CONCLUDING REMARKS

The clinical usefulness of DDP has been clearly established. Improvements in this drug can be made by increasing its efficacy, by decreasing its toxicity, or both. The results obtained to date suggest that the latter is truly an achievable goal and in many cases great strides have been made in diminishing host toxicity. Employing pharmacokinetic principles, chemoprotective agents and continued development of second or third generation platinum analogs should allow us to effectively separate many of the most troublesome toxicities associated with DDP.

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MECHANISMS OF RESISTANCE TO PLATINUM DRUGS

A. Eastman, N. Schulte, N. Sheibani and C.M. Sorenson

INTRODUCTION

Development of resistance to cancer chemotherapeutic agents is a major limitation to their clinical use. The phenomenon of resistance was encountered in the first documented case of cancer chemotherapy (1), and with an increasing ability to ameliorate drug-induced toxic side effects, resistance may now be the real limitation to providing a cure.

The study of resistance has two purposes: to determine whether development of resistance can be prevented, or whether resistance can be circumvented by appropriate therapeutic strategies. Considering that a tumor at the threshold for clinical detection contains 10^9 cells, it is perhaps to be expected that cells with different degrees of resistance already exist. Therefore, prevention of resistance is possibly an unattainable goal. However, an understanding of the mechanisms of resistance might provide a basis for overcoming this limitation. Success in this endeavor has been reported experimentally in the case of multiple resistance to many natural product antitumor drugs, often called pleiotropic drug resistance. The reduced accumulation of these drugs can be overcome by calcium channel blockers such as verapamil (2). For cis-diamminedichloroplatinum(II) (cis-DDP), the mechanisms of resistance are only now being elucidated and rational approaches to circumventing resistance are still in their infancy. This paper will review the observed patterns of resistance to platinum coordination complexes, relate this to potential mechanisms and finally describe the experiments from our laboratory that are elucidating these mechanisms of resistance.

CLINICAL RESISTANCE

The clinical definition of resistance is frequently that a tumor recurred after therapy. A more accurate definition would be that the recurrent tumor is refractory to the initial therapy. Most therapeutic regimes now include 3-5 different drugs which is an approach to circumventing resistance. Therefore, a recurrent tumor is resistant to a therapeutic regime not to a specific drug. Although resistance may be attributable to only one or two of the drugs, the complete regime becomes worthless unless the response to each individual component is evaluated. The ability to grow human tumor cells in soft agar (clonogenic assays) may help to clarify this situation even though many tumors cannot be evaluated by this method. In one report, a patient receiving cis-DDP, chlorambucil and 5-fluorouracil developed resistance only to the former two agents (3). In a more extensive study, prior treatment of ovarian cancer patients with cyclophosphamide, adriamycin, cis-DDP and 5-fluorouracil resulted in a generally reduced sensitivity to all the drugs although this was less marked for cis-DDP (4).

Resistance may also not be at the cellular level, rather alterations in pharmacodynamics could be involved. An observation that may be pertinent is that only some patients have immunologically detectable adducts in the DNA of their leucocytes after cis-DDP therapy (5). These nucleated blood cells are not the tumor, but the presence of DNA adducts appears to predict a therapeutic response. This suggests that a lack of response is not a property specific to the tumor cells and could reflect either enhanced drug clearance or enhanced DNA repair, both of which would reduce the number of DNA adducts.

EXPERIMENTAL RESISTANCE

Experimental cell systems resistant to cis-DDP abound. Murine leukemia L1210 and P388 cells were the first to be studied. Resistant cell lines were obtained by inoculating cells into mice and injecting cis-DDP over successive generations. Burchenal et al. (6,7) obtained L1210 cells up to 30-fold resistant to cis-DDP and some related analogues. Schabel et al. (8,9) developed similarly

resistant L1210 and P388 cells and additionally showed them to retain sensitivity to bifunctional alkylating agents such as melphalan. However, cells selected for resistance to melphalan were cross-resistant to cis-DDP.

Most subsequent studies have involved stepwise increase in drug concentration while the cells were grown in culture. This has permitted development of cis-DDP resistant human cell lines such as those of ovarian (10,11) and squamous cell carcinomas (12). Others have compared human bladder and prostate cells with different sensitivities, but these were unrelated cell lines and not selected for resistance (13,14). Another approach has been to compare normal cells to sensitive cell lines. Hence, cell lines deficient in DNA repair can be markedly sensitive to cis-DDP (15,16) whereas other sensitive cell lines such as the Walker 256 rat carcinoma showed no difference in DNA repair (17). This approach is of interest because cis-DDP is clinically most effective against embryological tumors and embryonal carcinoma cells in culture demonstrate enhanced sensitivity to cis-DDP (18). The emphasis in this paper will, however, be on cell systems with acquired resistance to cis-DDP.

Considerable insight can be obtained by analyzing patterns of cross-resistance to other drugs. We previously suggested that three patterns of cross-resistance reflect the majority of cases of cis-DDP resistance (19, Table 1). These patterns and the associated mechanisms of resistance appeared to relate to the drug used for selection. For instance, when human epithelial cells (20) or human ovarian carcinoma cells (21) were exposed to increasing concentrations of the heavy metal cadmium, the cells became resistant to cadium and cross-resistant with platinum compounds (category III).

Table 1. Patterns of resistance to platinum complexes

	Selection drug	Phenotype	Mechanism
IA	cis-DDP	cis-DDP^r DACH-Pt^s melphalan^s Cd^s	multiple
IB	DACH-Pt	cis-DDP^s DACH-Pt^r melphalan^s Cd^s	multiple
II	melphalan	cis-DDP^r DACH-Pt^r melphalan^r Cd^s	glutathione
III	Cd	cis-DDP^r DACH-Pt^r melphalan^r Cd^r	metallothionein

These resistant cells have an increased intracellular concentration of metallothionein which may inactivate the drug before it can produce the toxic lesion within the cell. Secondly, when murine leukemia L1210 cells were selected for resistance to the alkylating agent melphalan, they were found to be cross-resistant with cis-DDP (category II). Although initial studies suggested that a defective uptake mechanism may underly the resistance (22), more recent evidence suggests that this resistance is due to an increased level of glutathione which binds to and thus inactivates the drugs before they reach the critical DNA target (23). This mechanism of resistance may also be involved in a human Burkitt lymphoma cell line (Raji) selected for resistance to a chloroethylnitrosourea which was also cross-resistant to melphalan and cis-DDP (24). Not all melphalan-resistant cell lines are cross-resistant with cis-DDP.

A different pattern of cross-resistance is found in L1210 cells developed for resistance to cis-DDP by continual exposure to increasing concentrations of this drug (7,9). The resulting resistance is specific for cis-DDP and other closely related structural analogues (catagory I). These cells remain sensitive to 1,2-diaminocyclohexaneplatinum(II) complexes (DACH-Pt), melphalan, and cadmium. Although not tested against melphalan, a cis-DDP resistant but DACH-Pt sensitive Ehrlich ascites tumor probably fits this catagory (25). Similarly, when resistance was developed by continual exposure to DACH-Pt-carboxyphthalate, the cells displayed a lack of cross-resistance to cis-DDP, melphalan, and cadmium (26).

In categories II and III, cells generally attain only a modest level of resistance (2-7-fold) whereas, cells in category I can develop much higher levels of resistance. Hence, we have developed L1210 cell lines that are up to 100-fold resistant to cis-DDP (27,28). These cell lines, designated L1210/DDP, were developed by stepwise exposure to increasing cis-DDP concentrations over several years. The resistance is stable for long periods in the absence of drug. These cells exhibited a low level of cross-resistance to melphalan. A similar low level cross-resistance has been reported in a human ovarian carcinoma (11). The mechanism of resistance in these cells, as discussed below, may be complex involving alterations in drug accumulation, glutathione and DNA repair.

The variety of cross-resistance patterns discussed here emphasizes that different selection conditions can markedly influence the cell phenotype. This might suggest that resistance is not just selection of an existing altered cell because all possible patterns of resistance would be obtainable upon selection with cis-DDP. The stimulus for development of a particular type of resistance may depend upon the concentration of selecting drug. Hence, alterations in drug accumulation, glutathione or metallothionein may be adequate to facilitate low levels of resistance, but other mechanisms are required to increase resistance further. Enhanced DNA repair mechanisms are now recognized as contributing to these high levels of resistance (11; this paper).

THE INTERACTION OF PLATINUM COMPLEXES WITH DNA

The evidence that DNA is the critical target for cis-DDP is almost universally accepted. Our early studies investigated the formation and repair of DNA interstrand cross-links and DNA protein cross-links in sensitive and resistant L1210 cells (29,30). Neither of these lesions appeared to account for the majority of resistance in these cells. Accordingly, we set out to characterize all the DNA adducts that were produced by cis-DDP. These studies have recently been reviewed (31). For many of the studies we used a radiolabeled analogue, [^{3}H]-cis-dichloro(ethylenediamine)platinum(II) (cis-DEP). This facilitated detection of adducts and has proven invaluable in characterization of the adducts in cells (see below). The results of these studies showed that monofunctional adducts occur only transiently, with complete rearrangement to bifunctional adducts within 2 hr. About 90% of the adducts are intrastrand cross-links between neighboring bases on a DNA strand, either in GG or AG sequences (Fig. 1, 32-34). Other intrastrand cross-links may be separated by one intermediate base. Less than 1% of the damage is a DNA interstrand cross-link. Glutathione can also be cross-linked to DNA by cis-DDP (35).

Characterization of these adducts represents a preliminary step in determining the relative significance of each lesion to the therapeutic activity of the drug. Comparison with trans-DDP, the ineffective isomer of cis-DDP has often been used in attempts to

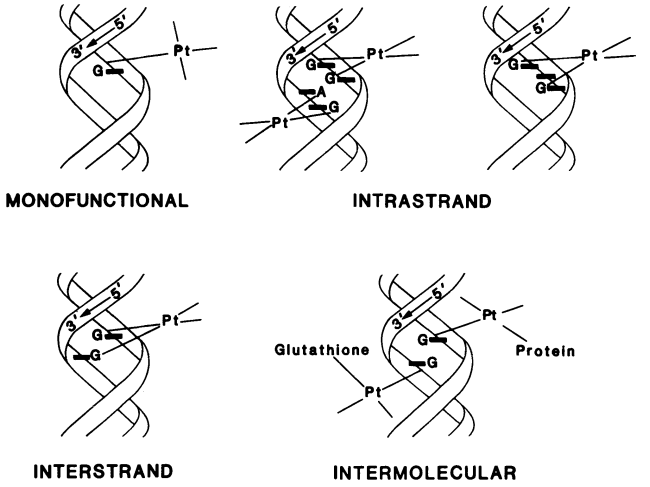


Fig. 1. Structures of the various adducts produced in DNA by cis-DDP (reproduced from reference 31 with permission of the copyright holder, Pergamon Journals Ltd., Oxford, England).

implicate various lesions. trans-DDP also reacts extensively with DNA to produce both interstrand cross-links and DNA-protein cross-links. However, it is sterically restricted in the type of intrastrand cross-links it could feasibly produce; that is, it is unable to cross-link neighboring bases in DNA. This has implicated intrastrand cross-links as important to the activity of cis-DDP. This hypothesis was not based on a knowledge of the adducts produced by trans-DDP; such an analysis has only recently been performed (36). The method involved in vitro incubations of DNA with trans-DDP, followed by enzyme digestion and analysis of the products by HPLC. Thiourea was used in an attempt to trap monofunctional adducts in DNA before they rearrange to bifunctional adducts. This strategy had previously been used successfully to measure the rapid rearrangement of cis-DDP adducts (34). Rather than trapping trans-DDP monofunctional adducts, thiourea labilized them from the DNA. At short time periods 85% of trans-DDP bound to double-stranded DNA as monofunctional adducts of deoxyguanosine. Rearrangement to bifunctional adducts was only 50% complete in 24 hr. The ineffectiveness of trans-DDP therefore results from a high proportion of monofunctional adducts in DNA that rearrange very slowly to toxic

bifunctional adducts. The persistent monofunctional adducts react rapidly with glutathione, which would further reduce their potential toxicity by preventing them from rearranging to more toxic bifunctional adducts. This presumably explains the observation that reducing glutathione levels in cells markedly enhances toxicity to trans-DDP but has little if any effect on cis-DDP toxicity (37).

RESISTANCE TO CIS-DDP IN L1210 CELLS

Cell lines

The development of the resistant cell lines has been previously reported (26-28). The characteristics of these cells are presented in Table 2. We have also investigated the genetics of resistance by making somatic cell hybrids between sensitive and resistant L1210 cells (28). Intermediate levels of resistance were obtained in these hybrids. As described below, this is explained by

Table 2. Properties of parental and hybrid cells

	IC_{50} (μg/ml)[a]		
Cell line	cis-DDP	DACH-Pt-SO_4	Melphalan
Parent cells			
L1210/0	0.09	0.22	0.64
L1210/DDP_2	2.1 (23)[b]	0.45 (2.0)	0.90 (1.4)
L1210/DDP_5	5.0 (56)	1.40 (6.4)	2.66 (4.8)
L1210/DDP_{10}	9.7 (108)	3.92 (18)	3.09 (5.6)
L1210/$DACH_4$	0.4 (4)	7.20 (33)	1.50 (2.3)
Hybrid cells			
O:DDP_5	1.55 (12)	0.73 (3.3)	2.16 (3.9)
O:DDP_{10}	1.90 (15)	1.32 (6.0)	1.40 (2.6)
O:$DACH_4$	0.60 (5.0)	0.98 (4.4)	1.32 (2.0)

[a] IC_{50} is the concentration effective in inhibiting 50% of cell growth measured after 3 days of continuous exposure to the drug.
[b] Values in parenthesis; fold resistance.

multiple mechanisms of resistance within a cell; one trait is recessive, another is dominant.

Drug accumulation

The cells and hybrids were investigated for possible alteration in accumulation of [^{3}H]-cis-DEP (28). All the resistant cells exhibited about 40% decrease in accumulation (Fig. 2). Reduced accumulation was therefore not proportional to the degree of resistance in these cells. Even the L1210/DACH cells which exhibited 6-fold cross-resistance to cis-DDP exhibited the same reduction in drug accumulation. We have not specifically addressed the question as to whether this phenomenon is related to altered uptake or efflux but, during the 24 hr after removal of drug, the resistant cells reduced the intracellular load by 80% compared to 70% for the sensitive cells (Fig. 2B).

The hybrid cells, when corrected for their increased cell volume, demonstrated accumulation levels close to that of L1210/0 cells (28). Reduced accumulation is therefore a recessive trait. Accumulation of DACH-Pt was also measured in these cells. The L1210/DACH cells demonstrated about 75% reduction in drug accumulation. This was also shown to be a recessive trait.

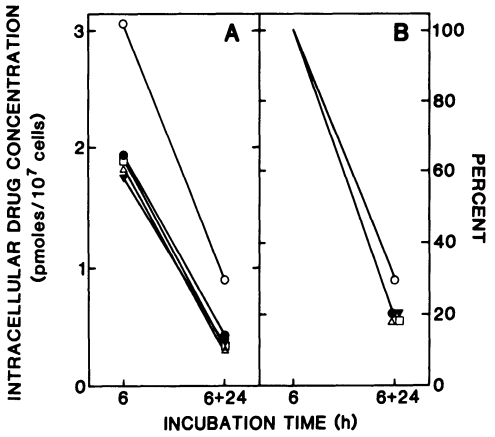


Fig. 2. Accumulation of [^{3}H]-cis-DEP after a 6 hr incubation with drug and the amount of drug retained after a further 24 hr incubation in the absence of drug. Results are expressed as (A) absolute concentration of drug and (B) as a percentage of that accumulated at 6 hr. The cell lines are L1210/0 (○), L1210/DDP$_2$ (▼), L1210/DDP$_5$ (□), L1210/DDP$_{10}$ (△), and L1210/DACH$_4$ (●).

Glutathione levels

The second mechanism of resistance examined was alteration in glutathione levels. Glutathione has been shown to be an important determinant of the sensitivity of cells to a wide variety of drugs (38). Elevated glutathione has also been shown to be a component of the drug-resistant phenotypes that emerge in cells exposed to a number of electrophilic drugs and especially to nitrogen mustards, such as melphalan (23). Melphalan-resistant L1210 cells were shown to be cross-resistant to cis-DDP (8). Glutathione levels in the cell could mediate platinum toxicity by at least two different mechanisms. Firstly, once the cis-DDP has entered the cell, it could directly bind to the sulfhydryl residues of glutathione forming a thioether. This would inactivate the drug before it could reach the critical DNA target. Secondly, glutathione may reduce cis-DDP toxicity by quenching DNA-Pt monofunctional adducts (35).

L1210/DDP_5 and L1210/DDP_{10} cells demonstrated a 1.7-fold increase in glutathione (28). The DACH-Pt-resistant cells and the hybrid cells did not exhibit this increase in glutathione levels, and, therefore, the increase in glutathione levels is also a recessive trait. Recently, we found that the elevation of glutathione is dependent upon the culture medium. Elevation was observed in McCoys 5A (modified) medium, but not in Minimal Essential Medium.

The role, if any, that glutathione plays in the resistance to cis-DDP is unknown. Our studies demonstrated that decreasing the intracellular glutathione levels in the resistant cells to that of the sensitive cells did not sensitize the cells to cis-DDP (28). Andrews et al. (39) have demonstrated that only after a prolonged decrease in the glutathione levels do the cells become sensitized to cis-DDP and that this is true for both sensitive and resistant cells. If the 1.7-fold increase in glutathione in the cis-DDP-resistant cell lines plays a role in the resistance to cis-DDP, it does not seem to be a simple correlation. It is worth noting that the cells with elevated glutathione are also more cross-resistant to melphalan. In this case, the glutathione may play a role in drug inactivation.

Metallothionein

Considering the potential of metallothionein to bind to and inactivate cis-DDP, we compared the sensitivity of the various cell lines to $CdCl_2$. Cells with elevated metallothionein would demonstrate resistance to $CdCl_2$. The L1210/0, L1210/DDP_{10} and L1210/$DACH_4$ cell lines exhibited IC_{50} values of approximately 70 μg $CdCl_2$/ml. Surprisingly, L1210/DDP_2 and L1210/DDP_5 were almost ten times more sensitive to $CdCl_2$. This obviates any role that metallothionein has in cis-DDP resistance in these cell lines.

DNA repair

The mechanisms of resistance so far discussed can only account for a minor degree of the resistance in these L1210 cells. The reduced accumulation and enhanced glutathione result in only a 30% reduction in the amount of [^{3}H]-cis-DEP bound to cellular DNA (Fig. 3A). The platination of cellular DNA was linear with drug concentration, therefore, the reduced DNA damage could contribute no more than 2-fold to the resistance. It was calculated that at the IC_{50} value for the L1210/0 cells (6 hr incubation with 0.5 μg cis-DEP/ml) there were 1.9×10^6 molecules of cis-DEP per cell and 1.9×10^4 molecules adducted to the DNA, i.e., 1 Pt/10^6 nucleo-

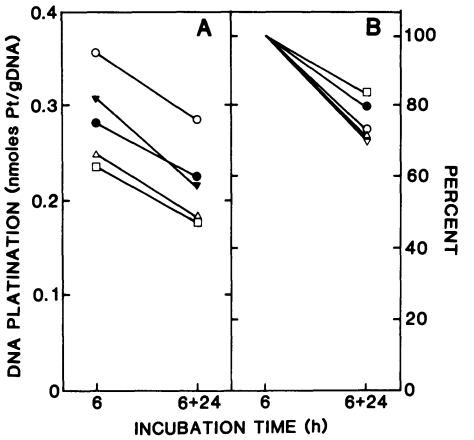


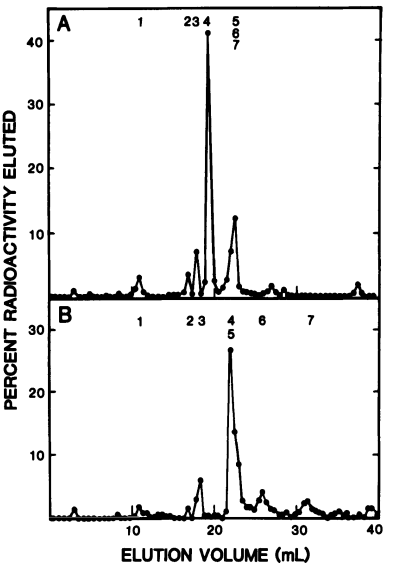
Fig. 3. DNA-bound radioactivity after incubation of L1210 cells with [^{3}H]-cis-DEP for 6 hr, and the amount retained in DNA after a further 24 hr incubation in the absence of drug. Results are expressed as (A) absolute platination of DNA and (B) as a percentage of that bound to DNA at 6 hr. Cell lines as in Fig. 2.

tides. At the IC_{50} value for L1210/DDP_{10} cells (6 hr incubation with 25 μg cis-DEP/ml) there were 9.5 x 10^7 molecules per cell and 9.5 x 10^5 molecules adducted to the DNA, i.e., 1 Pt/2 x 10^4 nucleotides. These values demonstrate that 1% of the intracellular drug is bound to DNA after a 6 hr incubation. Subsequently, DNA repair experiments were performed at non-toxic concentrations of drug. Twenty-four hr after platination, only 20-30% of the drug appeared to have been removed from the DNA in all the cell lines (Fig. 3B). These values have been corrected for DNA replication by the use of [^{14}C]thymidine in the assay. This also confirmed that all the cells had replicated during the 24 hr period.

The next step in the analysis required enzymatic digestion of the DNA and HPLC separation of the individual nucleoside-bound adducts. The HPLC profile of the adducts produced in L1210/0 cells is shown in Fig. 4. The use of ion suppression chromatography with different concentrations of elution buffer facilitated separation and characterization. Peaks 4, 6 and 7 represent adducts derived from GG, AG and GNG sequences, respectively. Peak 1 cochromatographed with a cross-link between deoxyguanosine and glutathione. Peaks 2, 3 and 5 were uncharacterized at this point. The profile of adducts from L1210/DDP_{10} cells showed only slight quantitative differences (not shown).

Cells were incubated in fresh medium for 24 hr after drug treatment. The profile of adducts in L1210/0 cells showed a slight reduction in peak 4 (Fig. 5). In contrast, the L1210/DDP_{10} cells showed almost complete loss of peak 4 and no detectable peaks 6 and 7. In this profile, peak 5 had become the major peak.

In an attempt to characterize these new peaks, they were purified by HPLC, incubated with 1 M thiourea and reanalyzed by HPLC. No alteration occurred in the chromatographic mobility of the radioactivity, suggesting that no platinum was present. On both reverse phase and cationic exchange columns, the radioactivity of peaks 3 and 5 cochromatographed with unmodified thymidine and deoxyadenosine. These peaks, therefore, result from dissociation of the drug, metabolism of [^{3}H]ethylenediamine and incorporation into DNA as the normal deoxyribonucleosides.



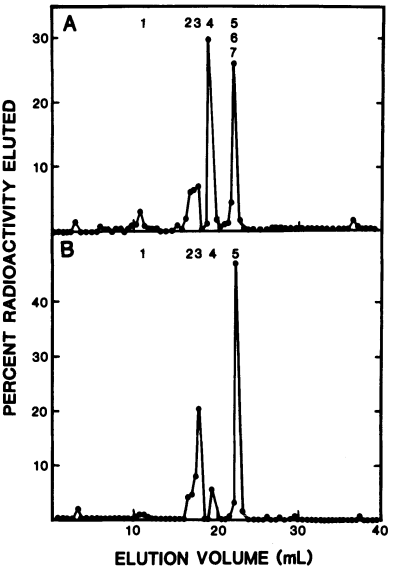


Fig. 4. (left) HPLC separation of DNA-bound adducts produced in L1210/0 cells incubated for 6 hr with [^{3}H]-cis-DEP. The HPLC elution buffer was (A) 0.2 M and (B) 0.02 M ammonium acetate. The identity of the numbered peaks is described in the text.

Fig. 5. (right) HPLC separation of DNA-bound adducts produced in (A) L1210/0 cells and (B) L1210/DDP$_{10}$ cells incubated with [^{3}H]-cis-DEP for 6 hr and incubated for a further 24 hr in drug-free medium. The HPLC elution buffer was 0.2 M ammonium acetate.

It was evident that the disappearance of peaks 4, 6 and 7 in these studies represented a real enhancement in a DNA repair process. To accurately quantify this process, we have analyzed specific [^{3}H]adducts from experiments in which cells were prelabelled with [^{14}C]thymidine. This obviated the contribution from both [^{3}H]nucleosides and DNA replication. The rate of repair of adducts at GG sequences was markedly enhanced in the resistant cells (Fig. 6). L1210/0 cells repaired 30% of these adducts in 6 hr while L1210/DDP$_{10}$ cells repaired 70%. Repair during the following 18 hr was considerably slower.

It should be emphasized that the rapid initial phase of repair will also occur during the 6 hr incubation with [^{3}H]-cis-DEP. Hence, the levels of platination observed will always be less than the amount of platination that actually occurred. The reduced level

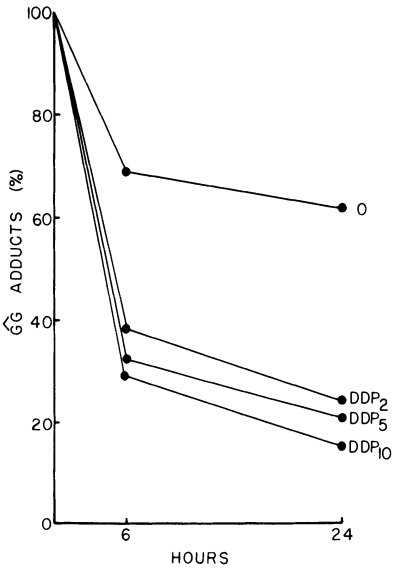


Fig. 6. Repair of DNA adducts at GG sequences in L1210/0, L1210/DDP_2, L1210/DDP_5 and L1210/DDP_{10} cells following a 6 hr incubation with [^{3}H]-cis-DEP (100% platination).

of DNA-platination in L1210/DDP cells discussed above and tentatively attributed to reduced accumulation of drug may, in fact, be a result of these differences in DNA repair rate. Accordingly, changes in drug accumulation may not contribute to resistance, rather altered DNA repair might be responsible for all of the observed resistance in L1210 cells.

While the above studies were being performed, an alternate assay for DNA repair was also being used. This assay involved platinating a plasmid DNA in vitro, introducing it into sensitive and resistant cells and measuring expression of a gene encoded by the plasmid. The plasmid used was pRSVcat which contains the bacterial cat gene coding for chloramphenicol acetyltransferase (CAT) in a configuration that permits expression in mammalian cells. Enzymatic activity of CAT is readily measured in cell lysates. Lesions introduced into pRSVcat will diminish cat gene expression after transfection. Repair of these lesions by the recipient cells will restore expression. This assay has previously been validated by the demonstration that normal human fibroblasts, but not

xeroderma pigmentosum fibroblasts, expressed ultraviolet-irradiated, transfected DNA (40,41). The plasmid DNA was incubated with different concentrations of cis-DDP and the level of platination confirmed by atomic absorption spectroscopy. The plasmid was transfected into L1210/0 cells and CAT activity assayed after 42 hr. The level of CAT activity decreased in a dose-dependent manner. A graphical representation of the results showed that a mean lethal hit (63% reduction in activity) occurred at a level of 8 platinum adducts per plasmid (Fig. 7). The CAT mRNA is 1640 bases, therefore, one-sixth of the plasmid DNA needs to be transcribed. The level of platination is, therefore, close to one cis-DDP adduct per transcribed gene and shows that every adduct was sufficient to totally inhibit expression. This suggests that the L1210/0 cells had little ability to repair the damage in the plasmid.

Transfection of cis-DDP-damaged plasmid into resistant cells showed that CAT activity was expressed at much higher levels of damage (Fig. 7). Quantitation indicated that, compared to the sensitive cells, up to eight times the amount of damage had to be introduced into the plasmid to produce a mean lethal hit. The

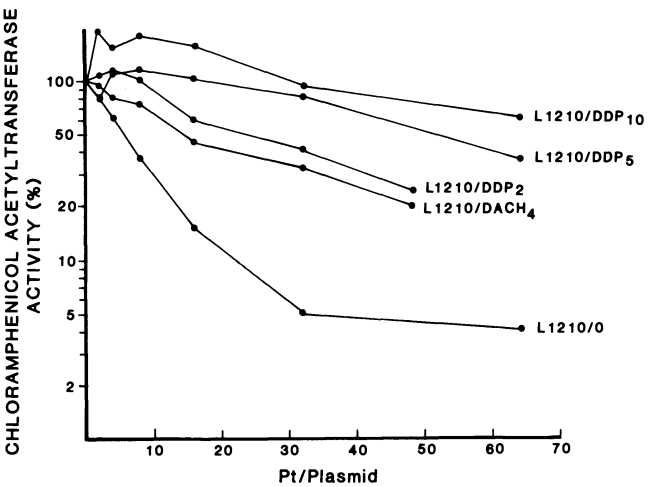


Fig. 7. Transient expression of the chloramphenicol acetyltransferase gene in L1210 cells transfected with cis-DDP-damaged pRSVcat.

hybrid cell lines also expressed CAT activity at levels close to those in the resistant cells, indicating that this mechanism of resistance is dominant.

Inhibitors of DNA repair

The experiments described in the preceding section clearly implicate alterations in DNA repair capacity as a major contributor to resistance in L1210 cells. Other results suggest this to be true in certain ovarian carcinoma cells (11). Therefore, it might be expected that inhibitors of DNA repair would resensitize these cells. It was recently reported that aphidicolin, an inhibitor of DNA polymerase α, enhanced the sensitivity of ovarian carcinoma cells to cis-DDP (42). We have investigated a DNA repair inhibitor that is already in clinical trials. Etoposide (VP-16) causes toxicity by interacting with topoisomerase II to produce protein-associated DNA breaks. L1210/DDP_{10} cells are 3-fold more sensitive to VP-16 than L1210/0 cells. This suggests that the enhanced DNA repair in L1210/DDP_{10} cells is associated with increased levels of topoisomerase. Although presumably necessary for resistance, enhanced levels of topoisomerase are not sufficient for resistance as evidenced by the lack of significant resistance to other platinum analogues and other DNA-damaging agents. The protein(s) that determines the specificity of resistance remains to be characterized.

These observations may have considerable clinical impact. In designing therapeutic regimes, it is advantageous if one drug overcomes resistance developed to another drug. If DNA repair is a common mechanism of resistance then VP-16 and cis-DDP may provide a synergistic combination. Evidence from animal models suggests these two drugs have synergistic activity (9). Clinical results have confirmed the efficacy of VP-16 in testicular cancer relapse after initial therapy that includes cis-DDP (43). This drug combination may also benefit patients with a variety of other tumors (44).

THE MECHANISM OF TOXICITY

Although DNA is almost universally accepted as the critical target for cis-DDP action, the mechanism by which such damage is translated into toxicity is unclear. It has frequently been

concluded that DNA synthesis is the most critical process because thymidine incorporation into DNA is reduced at lower concentrations of drug than are needed to reduce uridine incorporation into RNA (45-47). An attempt to correlate the extent of the inhibition of DNA synthesis with toxicity showed that much higher levels of drug were required to inhibit DNA synthesis than to inhibit cell growth (48). This experiment only measured DNA synthesis for 90 min immediately following drug treatment, whereas toxicity was monitored as growth over several days. We, therefore, investigated the possibility that the inhibition of DNA synthesis was a progressive event. In comparing cell doublings to DNA doublings over 3 days, it was evident that DNA replication continued even at concentrations which completely inhibited cell growth. Cells could almost double their DNA without an associated cell division.

These experiments were analyzed in greater detail by flow cytometry (Fig. 8). Generally, the same results were obtained with L1210/0 and L1210/DDP_5 cells at equitoxic concentrations. Cells were incubated with cis-DDP for 2 hr, incubated for a further 1-4 days and then analyzed for cell cycle distribution. There was a dramatic accumulation of cells in the G2 phase. At low drug

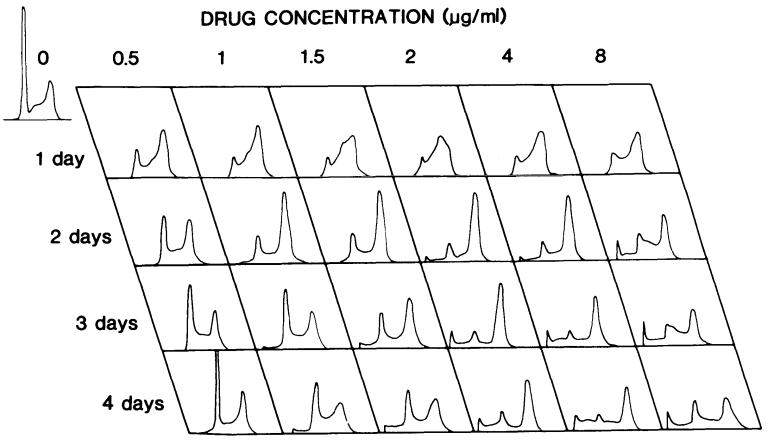


Fig. 8. Cell cycle analysis of L1210/0 cells incubated for 2 hr with the indicated concentrations of cis-DDP and then incubated for 1-4 days in drug-free medium. DNA content was assessed by flow cytometry.

concentrations, the G2 arrest was maximum at 24 hr, but was rapidly reversed. At higher concentrations, maximum G2 arrest occurred at 48 hr, demonstrating that the DNA synthetic phase was markedly slowed. This G2 arrest was also reversible except at the highest concentrations where cells began to die as shown by debris in the profiles. It was further shown by long-term growth curves that these cells regained growth potential even after a protracted arrest. It is concluded that two reasons exist for the observed inhibition of DNA synthesis; first, direct slowing of replication and second, synchronization of cells in the G2 phase. Neither of these events directly resulted in cell death.

We investigated the possibility that the G2 arrest was caused by the need to perform post-replication repair. This involves either filling gaps left in newly-synthesized daughter strands or recombination. We used the techniques of alkaline elution and neutral elution to measure single- and double-strand breaks, respectively. The formation of double-strand breaks correlated with the appearance of debris in the cell cycle profiles. No additional single-strand breaks were observed. It is concluded that post-replication repair occurs during, and is probably responsible for, the prolonged S phase. The observed breaks correlated best with toxicity.

A reason for the G2 arrest is still needed. It is known that transcription is required for passage through mitosis. In our DNA repair experiments reported above, it was shown that every adduct in DNA inhibited gene expression. This inhibition results in truncation of an mRNA followed by reinitiation. Although no qualitatively complete mRNA is made, little quantitative difference in transcription occurs. The G2 arrest occurs while cells repair DNA damage and recover the ability to make essential transcripts.

These results can be summarized in the following hypothesis: inhibition of transcription is more critical to toxicity than inhibition of DNA synthesis. Cells are obviously capable of replicating past lesions in DNA by processes collectively known as post-replication repair although the mechanisms are unknown. However, there is no known pathway whereby transcription can bypass lesions. Presumably at high concentrations of drug, the repair processes

required for recovery of transcription are themselves overwhelmed, endonucleases are expressed, DNA is degraded and cells die.

ACKNOWLEDGEMENTS

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HIGH DOSE CISPLATIN AND DRUG RESISTANCE: CLINICAL AND LABORATORY CORRELATIONS

R.F. Ozols, T.C. Hamilton, E. Reed, M.C. Poirier, H. Masuda, G. Lai and R.C. Young

INTRODUCTION

Cisplatin is a clinically important antineoplastic agent with particular usefulness in the treatment of testicular and ovarian cancer. The cure rate for patients with advanced testicular cancer has increased to 70-80% with cisplatin-based combination regimens (1). In patients with advanced ovarian cancer, cisplatin-based combination chemotherapy has increased complete response rates and prolonged overall survival compared to single agent therapy (2). However, due to the development of acquired drug resistance, the majority of patients with bulky ovarian cancer are not cured with standard dose cisplatin regimens. It has been shown that the dose of cisplatin is a critical factor in achieving optimum results in patients with either testicular or ovarian cancer. However, the toxicity of cisplatin (primarily nephrotoxicity) has prevented the routine administration of doses >100-120 mg/m^2.

In order to improve upon the effectiveness of cisplatin we have developed a comprehensive clinical and laboratory program aimed at: (1) identifying pharmacologic ways to improve the therapeutic index of cisplatin, (2) determining the mechanisms of cisplatin resistance with particular emphasis placed upon identification of pharmacologic techniques capable of reversing cisplatin resistance and (3) identifying clinical and laboratory parameters which may correlate with response to cisplatin therapy. These studies have been facilitated by the development of human ovarian cancer cell lines with different degrees of cisplatin resistance. These cell lines were developed by stepwise incubation of a drug sensitive cell line with increasing

concentrations of cisplatin (3). In addition, cell lines from cisplatin resistant patients have also been developed and one such cell line has been adapted for intraperitoneal growth in nude mice (OVCAR-3) (4).

MATERIALS AND METHODS

Administration of High Dose Cisplatin and High Dose Carboplatin

Based upon clinical observations of the importance of cisplatin dose in achieving optimum results as well as the steep dose response relationship of cisplatin in both cisplatin sensitive and resistant cell lines (see Fig. 1), we have been evaluating high dose cisplatin and high dose carboplatin in patients with testicular (5) and ovarian cancer (6,7). In these studies the dose of cisplatin is 200 mg/m^2 administered as 40 mg/m^2 i.v. qd x 5 while the dose of carboplatin is 800 mg/m^2 per cycle administered as 400 mg/m^2 i.v. qd x 2 by continuous infusion. The vehicle for high dose cisplatin is 250 ml of 3% saline while carboplatin was administered in D_5W. Hydration for high dose cisplatin consists of 6 liters/day of normal saline with

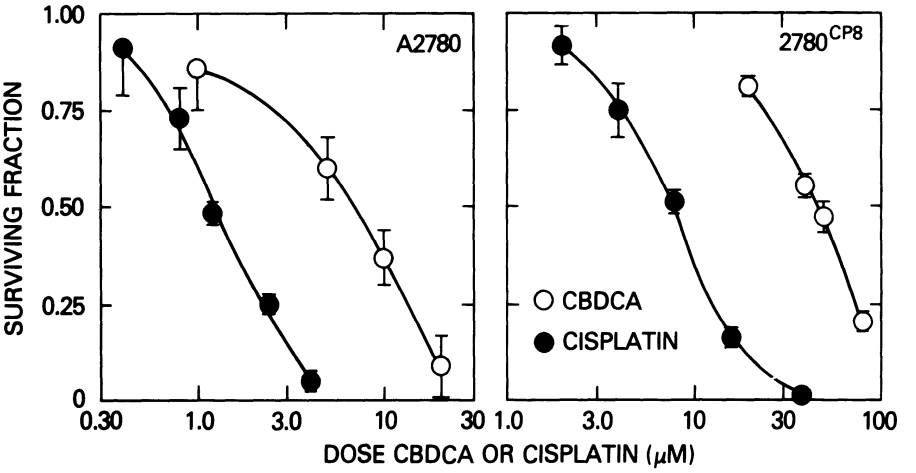

Fig. 1. Dose-response curve for cisplatin and carboplatin in the A2780 and 2780^{CP8} human ovarian cancer cell lines. ●, cisplatin and O, carboplatin [CBDCA].

20 meq of KCl per liter while standard hydration was used for carboplatin. In testicular cancer patients high dose cisplatin was administered as part of an aggressive regimen termed PVeBV (5) which consisted of cisplatin (P): 40 mg/m^2 i.v. qd x 5, vinblastine (Ve): 0.2 mg/kg i.v. on day 1, bleomycin (B): 30 units i.v. q week and VP-16 (V): 100 mg/m^2 i.v. qd x 5.

Patient Populations

Phase II trials of single agent high dose carboplatin and high dose cisplatin were performed in previously treated ovarian cancer patients. In addition, high dose cisplatin has been combined with cyclophosphamide in the induction therapy of previously untreated patients with advanced ovarian cancer in an ongoing clinical trial at the Medicine Branch. In testicular cancer, the PVeBV regimen has been used for the treatment of poor risk advanced nonseminomatous testicular cancer patients.

Collection of Specimens for DNA Platinum Adduct Formation

On the morning following completion of cisplatin and carboplatin therapy, 35-50 ml blood was obtained by venipuncture and placed in heparin ice tubes. The blood was centrifuged at 2000 rpm for 15 minutes and the "buffy coat" was aspirated and frozen at -20°C until DNA isolation. Measurement of N7-dGpG-dApG diammine platinum adducts in DNA isolated from the peripheral white cells of these patients was performed by an ELISA assay as previously described (8).

RESULTS

Clinical Studies in Ovarian Cancer Patients

Table 1 summarizes the results of high dose cisplatin and high carboplatin in previously treated ovarian cancer patients.

Table 1. Phase II Trials of High Dose Cisplatin and High Dose Carboplatin (6,7)

	CISPLATIN N=19	CARBOPLATIN N=27
RESPONSE TO THERAPY	No. (%)	No. (%)
Overall Response Rate	6 (32%)	8 (27%)
Partial Response	4 (21%)	4 (13%)
Clinical Complete Response	2 (11%)	4 (13%)
Minor Response	3 (16%)	3 (10%)
Progressive Disease	5 (26%)	12 (40%)
Disease Stabilization	5 (26%)	7 (23%)

On the basis of the results of the phase II trial of high dose cisplatin, a trial of high dose of cisplatin together with cyclophosphamide is currently in progress. The dose of cisplatin is 40 mg/m^2 qd x 5 and is administered together with cyclophosphamide 200 mg/m^2 qd x 5. Patients receive 3-4 cycles of therapy and then undergo restaging evaluation including second-look laparotomy if there is no clinical evidence of disease. The preliminary results of this ongoing trial are shown in Table 2 (9).

Table 2. High Dose Cisplatin + Cyclophosphamide and Whole Abdominal Radiation (CPR)

Patients (37)	No. (%)
Stage III	23 (62%)
Stage IV	14 (38%)
Response Category	
Overall Response Rate	30/37 (81%)
Clinical Complete	22/37 (59%)
Stage III	18/23 (78%)
Stage IV	4/14 (29%)
Partial Response	8/37 (22%)
NED at Laparotomy (PCR)	
After Chemotherapy	9/37 (24%)
After Chemotherapy + RT	2/37 (6%)
Total PCR	11/37 (30%)

High Dose Cisplatin in Testicular Cancer Patients

On the basis of a pilot study which demonstrated that PVeBV was effective therapy in previously untreated patients with high risk testicular cancer, a randomized trial of PVeBV versus standard PVeB chemotherapy in patients with poor prognosis germ-cell cancer is currently in progress at the Medicine Branch. Preliminary results of this trial have recently been reported and are summarized in Table 3 (10).

Table 3. Preliminary Results of PVeB vs PVeBV in Poor Risk Nonseminomatous Testicular Cancer

		SURVIVAL	
	CR	Overall	Disease-Free
PVeBV	30/34 (88%)	27/34 (79%)	26/34 (76%)
PVeB	12/18 (67%)	10/18 (56%)	8/18 (44%)
2-sided p	0.14	0.14	0.03

Toxicities of High Dose Cisplatin and High Dose Carboplatin

These studies demonstrate that high dose cisplatin and high dose carboplatin can be administered to patients with advanced ovarian and testicular cancer. The method of administration of high dose cisplatin as outlined in the Materials and Methods has eliminated nephrotoxicity as the dose limiting toxicity of platinum. We have described in detail the renal toxicity of high dose cisplatin (6,11). In 32% of advanced ovarian cancer patients there was a marked rise in serum creatinine (2.1-5.6 mg/dl) and a decrease in creatinine clearance with the first cycle of high dose cisplatin. However, renal function returned to normal in all patients over a 2-3 week period. The administration of subsequent cycles of high dose cisplatin was not associated with any clinically significant nephrotoxicity. Similarly, in testicular cancer patients there have been no dose modifications with cisplatin due to nephrotoxicity. High dose cisplatin has been administered even in patients who have obstructive uropathy due to retroperitoneal tumors.

The dose limiting toxicity of high dose cisplatin is neurotoxicity. Peripheral neuropathy developed in all advanced ovarian cancer patients who received more than 2 cycles of high dose cisplatin. Initial symptoms were numbness and tingling in the distal extremities and with 3 and 4 cycles of high dose therapy this frequently led to the development of severe neurologic abnormalities including gait disturbances. The profound decrease in proprioception led some patients to become temporarily wheelchair dependent. It is apparent that more than 3 cycles of high dose cisplatin cannot be routinely administered to patients with advanced ovarian cancer without the development of an unacceptably high rate of disabling peripheral neuropathy.

In contrast, the dose limiting toxicity of high dose carboplatin was the development of severe myelosuppression (7). The median white cell nadir in the phase II trial in advanced ovarian cancer patients was 0.6 and the median platelet nadir was 6,500 after the first cycle of high dose therapy. However, myelosuppression did not appear particularly cumulative. High dose carboplatin was not associated with any nephrotoxicity nor neurotoxicity.

Disease Response in DNA Platinum Adduct Formation

We have demonstrated that it is possible to measure DNA platinum adduct levels in the peripheral WBCs of patients undergoing therapy with either high dose cisplatin or carboplatin (12). The adduct level for each patient was compared to disease response. The 2-sided p value relating adduct level to disease response was 0.018 in the high dose cisplatin group and 0.020 in the high dose carboplatin group and 0.0015 when all patients were combined. With a retrospectively chosen cutoff of 160 attomoles of adduct per ug DNA used as a stratification factor, this level of adduct formation in WBC DNA also correlated with the response with a 2-sided p-value of 0.0013.

In Vitro Studies on Cisplatin Resistance

Table 4 compares the characteristics of the cisplatin sensitive cell line A2780 and the 2780^{CP} cisplatin resistant variant.

Table 4. Characteristics of A2780 and 2780^{CP}

Characteristic	A2780	2780^{CP}
IC_{50} for Cisplatin	1.1 μM	8.0 μM
Doubling Time (hours)	25.3	22.1
Cloning Efficiency (%)	28	36
Glutathione Content (nmol/10^6 cells)	4.46	10.7
Irradiation		
D_0	101	187
Extrapolation Number	1.4	1.6
Dose Modifying Effect with Buthionine Sulfoximine	4.3	3.2
Cross Resistance to Melphalan	-	2.1

It is of note that acquired resistance to cisplatin in 2780^{CP} is aasociated with cross-resistance to melphalan (3) and irradiation (13). The cytotoxic lesions produced by melphalan, radiation, and cisplatin are generally considered to be at the nuclear level through formation of DNA intra- and/or interstrand cross-links and/or DNA protein cross-links. We examined the effects of the DNA/reactive drugs on the induction of unscheduled DNA synthesis (UDS) as a measure of DNA repair. As can be seen in Figure 2, cisplatin and melphalan treatment of the drug resistant cell lines was associated with dose dependent increases in UDS while the A2780 sensitive cell line showed

essentially no capacity to repair DNA damage caused by cisplatin (3). Furthermore, we have also measured repair using cesium chloride gradients to separate repair synthesis from the semiconservative DNA replication (14). The specific inhibitor of DNA polymerase alpha (aphidicolin) was shown to inhibit DNA repair in a dose dependent manner with maximum inhibition at 4 ug/ml in the cisplatin resistant cell line (14). Evidence that this inhibition of DNA repair has implication for drug resistant ovarian cancer was demonstrated by the capacity of a non-cytotoxic dose of aphidicolin (10 ug/ml) to change the IC_{50} dose of cisplatin greater than 3-fold in the 2780^{CP} from 35 umol to 10 umol. Furthermore, at equitoxic doses of cisplatin which produced approximately a 20% reduction in clonogenicity in A2780 cell line (1 umol) and the cisplatin resistant cell line 2780^{CP} (20 umol), aphidicolin reduced survival in a dose-dependent manner to a level of less than 5% at 2 ug/ml for 2780^{CP}, but failed to have any impact on cisplatin cytotoxicity in A2780. These results demonstrate that inhibition of repair can partially reverse resistance to cisplatin in a cisplatin resistant cell line. In contrast, inhibition of DNA repair appears to be much less important in the expression of cisplatin cytotoxicity in a cisplatin sensitive cell line.

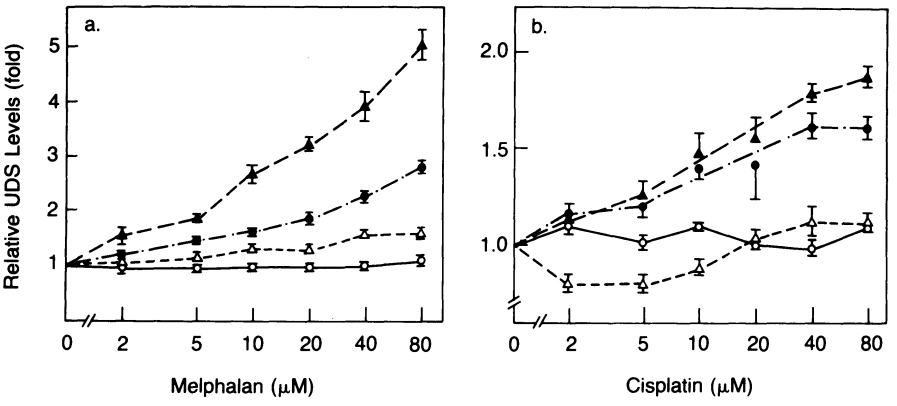

Fig. 2 Effect of melphalan (a) and cisplatin (b) on induction of UDS in human ovarian cancer cell lines A2780 (O), 2780^{CP} (▲), 2780^{ME} (●) and 2780^{AD} (Δ).

We have also demonstrated that reduction of glutathione levels in both the sensitive and resistant cell lines is associated with enhancement of the cytotoxicity of cisplatin, Table 4 (15).

DISCUSSION

The clinical studies demonstrate that high dose cisplatin and high dose carboplatin can be safely administered to patients with advanced ovarian and testicular cancer. The dose limiting toxicity of high dose cisplatin is peripheral neuropathy whereas the dose limiting toxicity of high dose carboplatin is myelosuppression. The clinical results also demonstrate a high degree of activity in relapsed ovarian cancer patients for high dose cisplatin and high dose carboplatin. However, in the absence of a prospective randomized trial, the role of high dose cisplatin in previously untreated ovarian cancer patients remains experimental. In testicular cancer patients, it is apparent that PVeBV is superior to PVeB in the treatment of high risk patients. However, the benefit of PVeBV may relate to the double-dose of cisplatin, the addition of VP-16 or to the synergistic affects of both agents.

Studies are in progress to determine if the dose limiting toxicities of platinum-containing compounds can be pharmacologically decreased and thereby permit further escalations of dose. Clinical trials have recently been initiated at the Medicine Branch using diethyldithiocarbamate in patients receiving high dose cisplatin and high dose carboplatin (9). Other studies are in progress to determine whether the radioprotective agent, WR2721, can decrease the neurotoxicity of cisplatin. It is also possible that colony stimulating factors may ultimately permit even higher doses of carboplatin to be administered since the only clinically significant toxicity of this agent appears to be hematologic suppression.

The observation that acquired resistance to cisplatin is accompanied by cross-resistance to other agents which cause DNA damage such as melphalan and radiation suggest that a common repair mechanism may be responsible, in part, for the primary resistance to cisplatin and for the cross-resistance to melphalan and radiation observed in 2780^{CP}. The demonstration that drug resistance can be

manipulated in these cell lines has potential clinical application. Buthionine sulfoximine has been shown to lower glutathione levels and potentiate the cytotoxicity of melphalan and platinum-containing compounds. While BSO also potentiates the toxicity of cisplatin, the LD_{10} dose of carboplatin and melphalan is not effected by co-administration of BSO to nude mice. Consequently, BSO plus melphalan and BSO plus carboplatin are possible treatment regimens in drug resistant patients and clinical trials await the completion of toxicology studies. In addition, the observation that aphidicolin can enhance cisplatin cytotoxicity in resistant cell lines will soon be evaluated clinically inasmuch as aphidicolin is currently undergoing phase I trials in Europe. And finally, the ovarian cancer cell lines have been useful to compare the cytotoxicities of platinum-containing compounds (3). In the cisplatin resistant cell line 2780^{CP} that there was marked cross-resistance to carboplatin, however, there is little cross-resistance to tetraplatin. Tetraplatin is a diamminocyclohexane platinum complex with a defined structure and sufficient purity, solubility, and stability for formulation and development and is currently undergoing toxicology testing prior to evaluation in clinical trials. Its marked activity in cisplatin resistant human ovarian cancer cell lines suggest that this compound may be of use in ovarian cancer patients who become resistant to cisplatin or carboplatin.

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COVALENT BINDING OF PLATINUM TO KIDNEY SUBCELLULAR FRACTIONS FROM GUINEA PIG STRAINS SENSITIVE OR RESISTANT TO CISPLATIN TOXICITY

C.L. Litterst and V.G. Schweitzer

INTRODUCTION

Since the introduction of platinum-containing anticancer drugs in the early 1970's scientists have attempted to find a mechanisnm by which the drug causes its toxic effects. Theories involving enzyme inhibition (1,2), DNA interaction (3) and immunomodulation (4) all have been proposed but each has limitations and shortcomings. In the past few years numerous drugs and chemicals have been found to exert their toxic effects through an irreversible binding to tissue components. Thus such disparate chemicals as bromobenzene, acetaminophin, and 4-ipomeanol all act through covalent binding to tissue proteins. Cisplatin, through its highly electrophilic chlorine centers has the potential to interact with tissue by such a mechanism and the prolonged biological half life of platinum in animals and humans, and the known binding of platinum to plasma proteins support this theoretical concept. It has recently been established that two strains of guinea pigs used widely in hearing research have an obvious difference in their response to cisplatinum. One strain (pigmented) is highly sensitive to cisplatin toxicity and the other strain (albino) is much more resistant to cisplatin toxic effects.

In an attempt to establish whether or not covalently binding of platinum to tissue might be an important mechanism of toxic action of cisplatin, we studied the covalent binding of platinum to proteins in subcellular fractions of kidneys from these two strains of guinea pigs following iv administration of cisPt. Our purpose was to discover if

there were differences in covalent binding between the two strains and if those differences correlated with differences in response to cisplatin.

METHODS

Female Hartley albino (Charles River Breed. Labs., Newfield, NJ) or NIH pigmented (Small Animal Section, VRB, NIH, Bethesda, MD) guinea pigs were anesthetized with 1:3 ketamine (100 mg/ml) to xylazine (10 mg/ml) im and the femoral vein surgically exposed. Cisplatin labelled with ^{195m}Pt (Oak Ridge National Laboratories, Oak

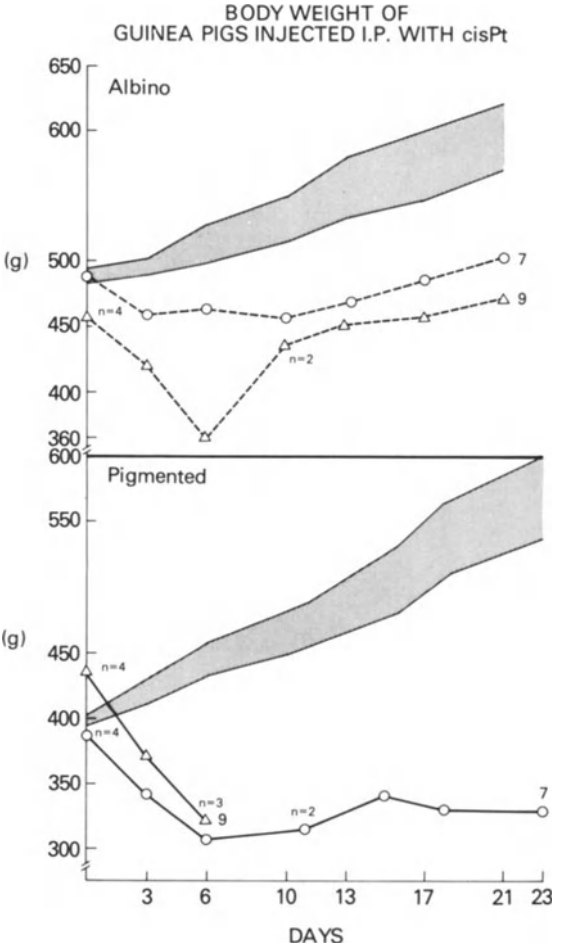

Figure 1.

Table 1. Tissue distribution of total platinum-derived radioactivity in 2 strains of guinea pigs (ug Pt/g tissue; X ± SD, N = 5)

	PIGMENTED			
	5 min	60 min	6 hr	24 hr
Kidney	65.6 ± 13.0	28.2 ± 0.6	14.6 ± 1.1	23.7 ± 0.3
Liver	13.2 ± 2.5	18.4 ± 1.0	10.6 ± 0.6	15.8 ± 0.2
Lung	21.2 ± 1.0	6.6 ± 0.7	4.7 ± 0.3	5.7 ± 1.0
Duodenum	10.3 ± 1.1	5.4 ± 0.1	3.8 ± 0.2	5.3 ± 0.1
Plasma	26.7 ± 2.0	6.1 ± 0.7	2.4 ± 0.2	3.3 ± 0.4
Urine	9 ± 2	---	20 ± 11*	26 ± 12
	ALBINO			
	5 min	60 min	6 hr	24 hr
Kidney	78.8 ± 12.4	32.2 ± 1.9	26.8 ± 1.3	18.6 ± 1.0
Liver	18.3 ± 1.1	20.6 ± 1.9	16.9 ± 0.3	12.0 ± 2.5
Lung	31.7 ± 2.5	6.5 ± 1.7	6.8 ± 0.6	6.2 ± 0.6
Duodenum	12.6 ± 1.1	5.3 ± 1.9	4.4 ± 0.6	4.1 ± 0.5
Plasma	32.7 ± 1.1	5.8 ± 0.5	3.6 ± 0.1	2.5 ± 0.1
Urine	7 ± 2	----	39 ± 3*	44 ± 4

*4 hour collection

Ridge, TN; 45 mCi/mg) was administered through the femoral vein and the incision closed with wound clips. At various times (5 min, 60 min, 6 hr, 24 hr) afterward groups of animals (n = 5) were killed by decapitation and tissues removed and analyzed for radioactivity. The 24 hr animals were placed in metabolic cages to allow determination of urinary excretion of radioactivity. Subcellular fractions of kidney were isolated by homogenizing the tissue (1:10) in 0.25 M sucrose containing 50 mM tris HCl (pH 7.4) and the resulting homogenate centrifuged for 10 min at 1000 x g to sediment cell debris and nuclei. The supernatant was then centrifuged for 20 min at 9,000 x g

to sediment mitochondria (MITO) and the supernatant further centrifdiged for 60 min at 105,000 x g to obtain a microsomal pellet (MICRO) and the cytosol (CYTO). Total radioactivity and total protein (5) were determined on each subcellular fraction. Each fraction then was treated with 20% trichloroacetic acid (TCA) and the resulting acid-insoluble protein pellet exhaustively extracted by washing with 5% TCA, warm (60°) methanol (MeOH), 5% TCA, MeOH, 5% TCA, and then 5 additional MeOH washes. Radioactivity was determined in each wash. After dissolving the final pellet it 1N NaOH at 80° C for 20 min, protein and radioactivity were determined. This was considered to be radioactivity that was covalently bound to tissue protein. Extraction efficiency was established by determining the radioactivity in all of the acid or alcohol washes. Radioactivity in washes was at background levels by the second or third of the final 5 alcohol washes for all subcellular fractions. Correction for radioactive decay was made by counting a sample of treatment solution at the time of treatment and again each time sample radioactivity was determined. Data are expressed as X ± SD of total platinum present per mg of protein.

Table 2. Distribution of radioactive platinum in subcellular fractions of guinea pig kidney prior to extraction (ng Pt/ mg Protein; X ± SD, n = 5).

	PIGMENTED		
	MITO	MICRO	CYTO
5 min	88 ± 17	112 ± 25	673 ± 198
60 min	113 ± 9	148 ± 11	228 ± 18
6 hr	113 ± 7	119 ± 11	142 ± 6
24 hr	158 ± 9	109 ± 6	129 ± 9
	ALBINO		
	MITO	MICRO	CYTO
5 min	114 ± 9	170 ± 15	851 ± 68
60 min	136 ± 11	152 ± 3	270 ± 27
6 hr	149 ± 11	133 ± 9	194 ± 4
24 hr	130 ± 8	138 ± 7	143 ± 10

RESULTS

Differences in sensitivity of the two guinea pig strains to cis-platin injected ip at two doses are shown in Figure 1. Pigmented animals exhibited greater mortality at lower doses or died at earlier times than was true for the albino animals. Studies of multiple small iv injections (1 mg/kg) also showed the pigmented guinea pigs to be more sensitive. Histopathologic evaluation showed equal renal necrosis in albino and pigmented animals (Data not shown).

Table 1 shows distribution of radioactive platinum in tissues and fluids of both strains at various times after drug administration. Tissue distribution is similar in both strains, as is the apparent time course of decay. Recovery of radioactivity in urine, however, was much greater in albino than in pigmented animals thoughout the 24 hr time course.

The concentration of platinum in each subcellular fraction prior to extraction is shown in Table 2 and is similar for the two strains of animal. Thus concentrations of platinum are initially highest in CYTO and continue to be high in that fraction through the first 6 hr after treatment. By 24 hr after dosing, platinum levels in all fractions of albino animals are equal, and levels in MICRO from pigmented animals are only minimally less than in CYTO or MITO.

Table 3. Distribution of covalently bound platinum in subcellular fractions of guinea pig kidney (ng Pt/mg protein; X ± SD)

	PIGMENTED		
	MITO	MICRO	CYTO
5 min	39 ± 5	68 ± 11	66 ± 14
60 min	118 ± 9	145 ± 11	216 ± 17
6 hr	108 ± 6	120 ± 11	144 ± 6
24 hr	140 ± 6	101 ± 5	122 ± 8
	ALBINO		
	MITO	MICRO	CYTO
5 min	49 ± 9	98 ± 8	95 ± 10
60 min	129 ± 10	186 ± 12	125 ± 6
6 hr	119 ± 5	169 ± 10	114 ± 5
24 hr	95 ± 4	121 ± 6	75 ± 1

After exhaustive extraction amounts of platinum covalently bound to tissue protein appear initially to be greater in albino than in pigmented animals (Table 3). Thus in albinos, 50% more platinum is found in CYTO and MICRO than is true for similar fractions from pigmented animals 5 min after treatment. By 60 min, however, fractions from pigmented guinea pigs contain equal or greater amounts of covalently bound platinum than do similar fraction from albino guinea pigs, an effect which is generally true throughout the remainder of the 24 hr study. Thus if one plots the rate of decay of CYTO platinum in the two strains, the half times of platinum in pigmented guinea pigs is consistently greater for pigmented than for albino animals (Table 4).

When one examines the covalently bound platinum as a percent of total platinum in each fraction, it is apparent that binding in pigmented animals differs from that in albinos (Table 5). Thus in CYTO of pigmented animals nearly 100% of total platinum is covalently bound beginning at 1 hr , while in albino animals, only 45-60% of total platinum is covalently bound. A similar results is seen in MITO, but not in MICRO. The protein to which the platinum is bound so extensively and avidly is unknown and we do not yet understand the relation between highly bound platinum and the greater sensitivity of pigmented relative to albino guinea pigs.

Table 4. Percent of total platinum covalently bound to kidney subcellular fractions

	PIGMENTED		
	MITO	MICRO	CYTO
5 min	44	61	9.8
60 min	104	98	95
6 hr	96	101	101
24 hr	88	93	95
	ALBINO		
	MITO	MICRO	CYTO
5 min	43	58	11.1
60 min	95	122	46
6 hr	80	127	59
24 hr	73	88	52

Table 5. Estimated half times of platinum in kidney subcellular fractions (hours)

	TOTAL PLATINUM	
	PIGMENTED	ALBINO
CYTO	186	33
MICRO	84	flat
MITO	increasing	75

	COVALENTLY BOUND PLATINUM	
	PIGMENTED	ALBINO
CYTO	50	33
MICRO	56	35
MITO	increasing	57

DISCUSSION

It is apparent from these studies that a greater percent of platinum is covalently bound in pigmented guinea pig CYTO and MITO than is true in similar fractions from albino guinea pigs, and that the kinetics of removal of that covalently bound drug is different between the two strains, with removal being slower from pigmented than from albino animals. This latter fact suggests that the binding protein may be different between the two strains, because one would expect catabolism and replacement of specific, individual proteins to be similar between strains. In addition, the total amount of platinum bound covalently in CYTO is greater for 1-24 hr in pigmented than in albino animals.

However, we do not yet understand the relation between renal covalently bound platinum and lethality in these--or any other--strains of animals. It is possible that animals die from non-renal mechanisms, although the time course of death is similar to that observed in rats, which die with extensive renal lesions, and the pigmented guinea pigs have BUN values which are greater at 24 hr than BUN values from albino

guinea pigs (113 ± 10 vs 75 ± 16 mg/dl). However, renal necrosis is similar between the two strains, althoughthe gastrointestinal lesions may be greater in the pigmented animals (Data not shown).

The very high concentration of platinum present initially in CYTO of both strains and which is not covalently bound may represent binding of platinum to low molecular weight, acid-soluble components of cytosol, such as glutathione or metallothionein, both of which have been reported to bind platinum (6,7). The high levels of radioactive platinum in CYTO also may represent non-metabolized parent drug that is subsequently excreted in the interval before the next time point at 1 hr. Rapid renal excretion of parent drug has previously been postulated(8,9).

The significance of platinum binding in MITO has not been fully appreciated by investigators. Electron microscopic evaluation of early renal cellular changes routinely involves changes in size or shape of mitochondria (10,11). In addition, it is obvious that the amount of platinum covalently bound to mitochondria in this study is higher in pigmented guinea pigs than in albinos and remains higher throughout the time period of 1-24 hr. Again, the correlation between covalent binding and toxicity is apparent, but the causal relation is less obvious.

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INHIBITION OF PLATINUM DRUG TOXICITY BY DIETHYLDITHIOCARBAMATE

R.F. Borch, P.C. Dedon, A. Gringeri and T.J. Montine

INTRODUCTION

The toxicity of platinum antitumor drugs constitutes the major impediment to the optimal use of these agents in the clinic (1). Numerous organs are affected by the platinum drugs, and the site of dose-limiting toxicity varies. Cisplatin nephrotoxicity is dose-dependent and has generally been dose-limiting. Numerous strategies have been developed to reduce kidney damage, however, and neurotoxicity currently limits the total cisplatin dose that can be administered using standard hydration-diuresis protocols. Carboplatin is significantly less toxic than cisplatin to kidney, GI tract, and peripheral nerves, but its use is currently limited by the profound myelosuppression observed with high dose regimens. Recent efforts to ameliorate these toxicities have focused on the development of "second drugs", or chemoprotectors, that can react with and inactivate the species responsible for the toxic effect. Most of these compounds contain a nucleophilic sulfur moiety, based upon the known affinity of sulfur-containing ligands for platinum (II) complexes. Selectivity is a potentially serious problem for these compounds, because reaction with the platinum drug or its metabolites may also inhibit the cytotoxic reaction in the tumor cell. This is particularly true for those protectors (WR-2721 and thiosulfate, for example) that must be administered just prior to or concomitant with the platinum drug. Here selectivity may depend upon preferential concentration in host tissue vs. tumor cells. Our initial approach with diethyldithiocarbamate (DDTC) was based upon a "rescue" mechanism, with DDTC given at a fixed

time after the platinum drug. According to this hypothesis, the platinum drug would distribute and bind to targets in both normal and tumor cells and, because of mechanistic differences in the drug-target interactions, DDTC would reverse platinum binding in normal cells without interfering with the DNA platination reactions in tumor cells (2-4). We have shown that DDTC inhibits cisplatin toxicity to the kidney, gut, and bone marrow and also reduces the myelosuppression of carboplatin. DDTC is also the only chemoprotector that is effective when given after the platinum drug.

CHEMISTRY

The biologic activity of the platinum (II) antitumor agents is presumably governed by ligand exchange reactions occurring with a variety of nucleophiles. Reaction with nucleophilic species of relatively low reactivity occurs via first-order rate-limiting displacement of chloride or carboxylate leaving groups by water. Subsequent exchange of water ligand and nucleophile leads to rapid product formation. For the highly reactive sulfur nucleophiles, a second-order reaction involving direct displacement of the chloride or carboxylate leaving group can also occur. This reaction rate will be dependent upon nucleophile concentration and will generally be competitive with the aquation-exchange pathway only for thiol concentrations in the millimolar range.

The reaction kinetics of several platinum complexes with thiols were measured in order to evaluate the likely importance of platinum-thiol reaction rates to chemoprotector efficacy and glutathione (GSH) inhibition of toxicity and antitumor effect. The second-order rate constants and the estimated reaction half-lives at physiologic or pharmacologic nucleophile concentrations are summarized in Table 1. These rate constants vary over many orders of magnitude and are dependent upon nucleophile structure and platinum complex ligand substitution and geometry. In general, thiols react several hundred fold more slowly with cisplatin than with the trans isomer and approximately 10-fold more slowly with carboplatin than with cisplatin. DDTC reacts 5-8 times faster with the Pt(II) complexes than does GSH.

Table 1. Second-order rate constants and estimated half-times for platinum complex substitution reactions (pH 7.4, 37°) (adapted from ref. 2)

Reactants	$k_2 \times 10^4$ $(M \cdot s)^{-1}$	$t_{1/2}$ (min)[a]
CISPLATIN		
Water	0.02	105 (55 M)
Glutathione	132	174 (5 mM)
Cysteine	386	5900 (50 uM)
Methionine	396	5800 (50 uM)
Thiosulfate	570	202 (1 mM), 10 (20 mM)
DDTC	614	187 (1 mM)
CARBOPLATIN		
Water	0.00018	11,500 (55 M)
Glutathione	9.2	2500 (5 mM)
Thiosulfate	85	1350 (1 mM), 68 (20mM)
DDTC	76	1510 (1 mM)
IPROPLATIN		
Water	0.0007	2960 (55 M)
Glutathione	6.0	3800 (5 mM)
DDTC	6.6	17,400 (1 mM)
TRANS-DDP		
Water	0.074	28 (55 M)
Glutathione	39,100	0.59 (5 mM)
DDTC	255,000	0.45 (1 mM)
$Pt(GSH)_2$		
Thiosulfate	< 1	$> 10^6$ (1 mM)
DDTC	499	230 (1 mM)

[a] nucleophile concentration in parentheses.

The short half-time (< 1 min @ 5 mM GSH) for the reaction of trans-DDP with GSH may account for the rapid inactivation and decreased cytotoxicity of this platinum complex. It is interesting to compare the reaction rates of DDTC and thiosulfate, an agent that inhibits cisplatin nephrotoxicity when given simultaneously. DDTC and thiosulfate react at comparable rates with cisplatin and carboplatin; however, the respective half-lives of 3 and 24 hours at pharmacologic concentrations imply that, in the absence of a mechanism to concentrate the chemoprotector, the direct reactions of DDTC and thiosulfate with platinum drug are too slow to account for the protection observed. In contrast, the reaction rates of thiosulfate and DDTC with platinum coordinated to a thiol ligand are very different. DDTC reacts with the cisplatin-GSH complex at a rate comparable to that for cisplatin, and it restores the enzyme activity of cisplatin-inhibited rat kidney g-glutamyl transpeptidase (GGT) in vitro. Thiosulfate is essentially unreactive toward both the cisplatin-GSH complex and cisplatin-inhibited GGT (Fig. 1)(2).

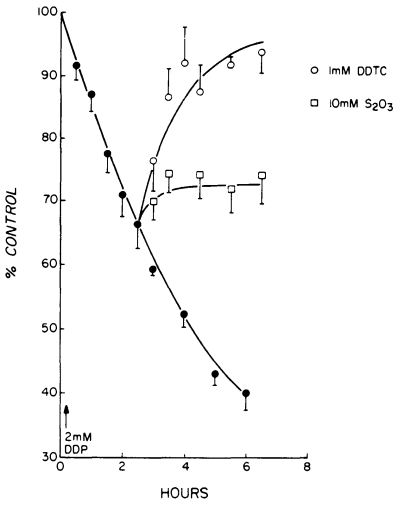

Fig. 1. Inhibition of rat GGT by 2 mM DDP (●) and reactivation by DDTC (○) or by thiosulfate (□).

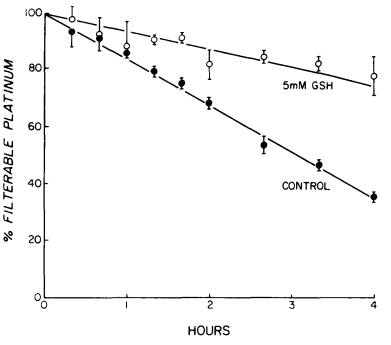

Fig. 2. Inhibition of DDP binding to salmon testes DNA by glutathione (5 mM).

It has been suggested that elevated levels of intracellular GSH may contribute to decreased platinum drug toxicity in normal cells and to increased platinum drug resistance in tumor cells. The parent platinum drugs react very slowly with GSH, however; in the presence of 5 mM GSH, cisplatin has a half-life of almost 3 hr. Thus it is unlikely that the effects of GSH on platinum drug cytotoxicity result from direct reaction between GSH and parent drug. The rate of cisplatin binding to salmon testes DNA decreased to 25% of control value in the presence of 5 mM GSH (Fig. 2), confirming that GSH can intercept and inactivate reactive platinum intermediates.

NEPHROTOXICITY

The current hypothesis regarding the unique property of DDTC to inhibit platinum drug toxicity in normal tissues is based upon the ability of DDTC to remove platinum from sites other than guanine cross-links in DNA (3,4). Implicit in this hypothesis is the assumption that either 1) DNA is not the cytotoxic target in normal tissues, or 2) if DNA is the target, the platinum drug has not yet formed a bis guanine complex at the time of DDTC treatment. We are currently using the LLC-PK1 cell line as an in vitro model for studying the mechanisms of cisplatin nephrotoxicity and DDTC protection. The LLC-PK1 line is an immortal epithelial cell line derived from porcine kidney that expresses many functions characteristic of proximal tubule cells when grown to a confluent monolayer (5). Cell viability was measured by lactate dehydrogenase activity, total protein adherent to the culture plate, or by trypan blue exclusion; consistent and reproducible results were obtained with all three methods. Treatment of LLC-PK1 monolayers with cisplatin (200-400 uM, 1 hr) had no effect on viability 2 hr (> 95%) or 12 hr (> 90%) after treatment. Viability subsequently decreased in a time- and dose-dependent manner; at 72 hr,it was 71%, 35%, and 10% of control values, respectively, after 1-hr exposure of cells to 200, 300, and 400 uM cisplatin (Fig. 3). Trans-DDP was approximately 5-10 fold less toxic in this viability assay, and the non-nephrotoxic

analogs carboplatin and iproplatin showed no reduction in viability when cells were treated at concentrations up to 2 mM for 1 hr. Cisplatin toxicity appears to be dependent upon the product of drug concentration and exposure time; loss of viability was identical when drug concentration was doubled and exposure time halved.

Both thiosulfate and DDTC significantly increased the viability of cisplatin-treated LLC-PK1 cells, and the time dependence of thiosulfate protection was equivalent to that observed in vivo (Figs. 3 and 4) (6). Treatment of cells with thiosulfate or DDTC (1 mM, 1hr) just before cisplatin exposure was highly effective in preserving the 72-hr viability of the cells. DDTC but not thiosulfate protected viability when treatment followed cisplatin exposure. Treatment with 3mM DDTC was marginally more effective than with 1 mM DDTC (97% vs 86% @ 200 uM, 85% vs 72% @ 300 uM, and 42% vs 34% @ 400 uM cisplatin, respectively). Thus it appears that both DDTC and thiosulfate enter the LLC-PK1 cells and block the toxic reaction when present prior to cisplatin treatment. This result is not surprising given the equivalent reactivities of DDTC and thiosulfate toward cisplatin and potential toxic metabolites. However, DDTC is uniquely able to reduce cisplatin toxicity when used after cisplatin. These results are consistent with the protective effects of thiosulfate and DDTC observed in vivo and demonstrate the utility of this cell line as an appropriate in vitro model for platinum drug nephrotoxicity.

We have also measured total platinum and platinum-DDTC complex in these cells using AAS and HPLC assays. Intracellular platinum concentrations were 120 and 190 uM following 1-hr treatment with 200 and 400 uM cisplatin, respectively. Subsequent treatment with DDTC (3 mM, 1 hr) had no effect on intracellular platinum concentration. The platinum-DDTC chelate represented 10-20% of total platinum in these cells, confirming that this complex can be formed intracellularly in the proximal tubule cell model.

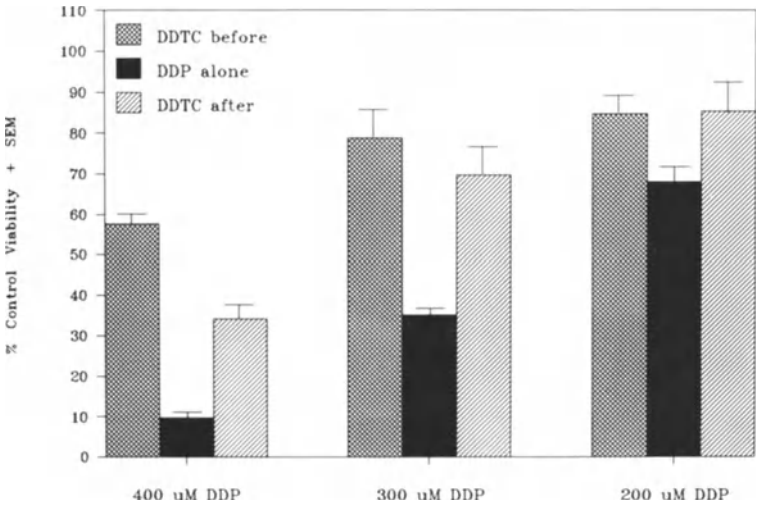

Fig. 3. Viability of LLC-PK1 cells after treatment with cisplatin (200-400 uM, 60 min) with or without DDTC (1 mM, 60 min) before or after cisplatin. Viability measured 72 hr after cisplatin treatment.

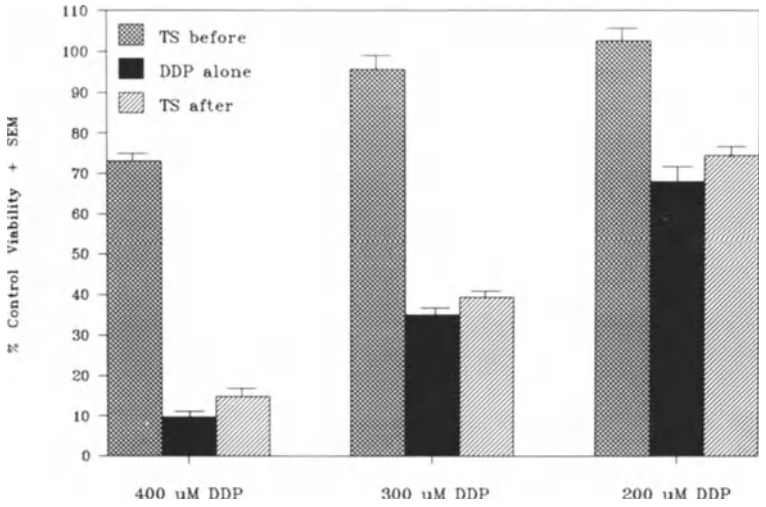

Fig. 4. Viability of LLC-PK1 cells after treatment with cisplatin (200-400 uM, 60 min) with or without thiosulfate (1 mM, 60 min) before or after cisplatin. Viability measured 72 hr after cisplatin treatment.

MYELOSUPPRESSION

We had shown previously that treatment with DDTC following cisplatin administration in mice reduced the toxicity to the granulocyte but not the lymphocyte population in the bone marrow (4,7). DDTC increased the nadir and decreased the subsequent overshoot of granulocyte counts and also provided more rapid bone marrow recovery. The ability of DDTC to inhibit the myelosuppression of both cisplatin and carboplatin in mice has been confirmed using the CFU-S assay (8). BDF_1 mice were treated with iv cisplatin or carboplatin with or without subsequent DDTC treatment. Marrow cells were harvested 24 hr later and injected into lethally irradiated mice. Spleen colonies were counted after 10 days. Both cisplatin and carboplatin produced a dose-dependent reduction in colony counts; cisplatin was somewhat more toxic than carboplatin to stem cells at an equimolar dose (15% of control @ 32 mg/kg cisplatin vs. 26% of control @ 40 mg/kg carboplatin). DDTC rescue significantly reduced both cisplatin and carboplatin toxicity to the marrow stem cells at all platinum drug doses tested (Figs. 5 and 6). This treatment provided a dose modification factor of 3-4 compared with the platinum drugs alone. The reduction of toxicity was essentially independent of DDTC dose from 100-750 mg/kg and independent of platinum drug-DDTC interval from 1-3 hr for cisplatin and 1-5 hr for carboplatin. The clonogenic CFU-GM assay was used to assess the effects of platinum drug treatment with or without DDTC rescue on the committed granulocyte/macrophage progenitor cells. Mice were treated as outlined for the CFU-S assay, and bone marrow cells were harvested and plated 24 hr after drug treatment. Granulocyte/macrophage colonies were counted after 12 days. Cisplatin toxicity to the G/M progenitor cells was somewhat less than that to the stem cells; DDTC was less effective as an inhibitor of G/M toxicity (Fig. 7). The G/M progenitor cells were significantly less sensitive than the stem cells to carboplatin toxicity (Fig. 8), and the protective effect of DDTC to the G/M population was apparent only at the highest carboplatin dose tested. These data suggest that carboplatin is primarily toxic to the stem cell population in the

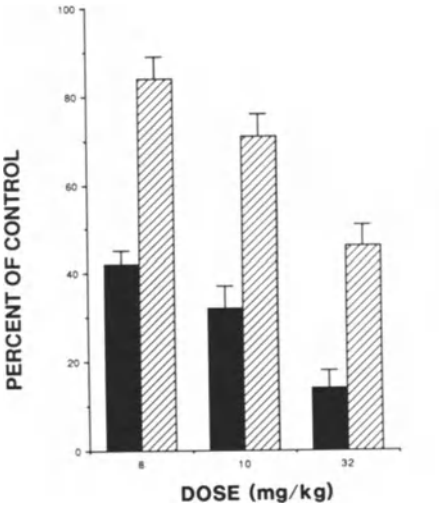

Fig. 5. CFU-S following iv cisplatin with (▨) or without (■) DDTC (300 mg/kg iv) 2 hr later.

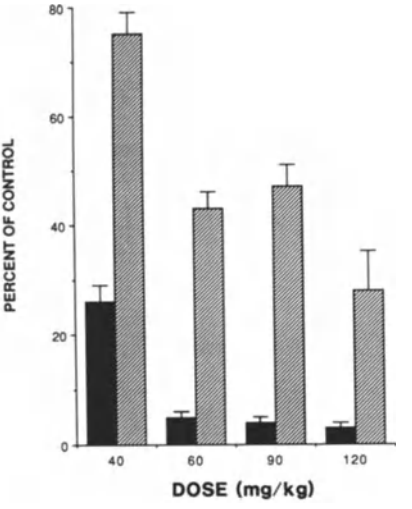

Fig. 6. CFU-S following iv carboplatin with (▨) or without (□) DDTC (300 mg/kg iv) 3 hr later.

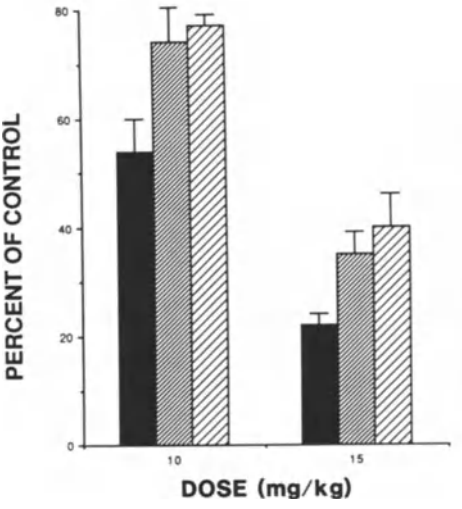

Fig. 7. CFU-GM following iv cisplatin and DDTC 2 hr later: DDTC 0 (■); 300 (▨); or 500 (▨) mg/kg.

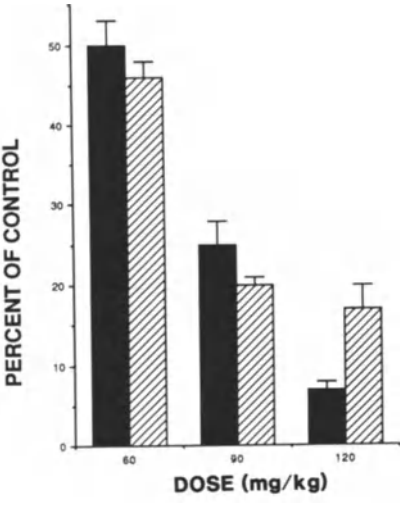

Fig. 8. CFU-GM following iv carboplatin with (▨) or without (■) DDTC (300 mg/kg iv) 3 hr later.

bone marrow, and this may account for the prolonged myelosuppression that occurs clinically with high-dose carboplatin administration. The efficacy of DDTC as an inhibitor of carboplatin toxicity to the marrow stem cell population is very encouraging and may permit substantial dose escalation of carboplatin in the clinic.

CLINICAL STUDIES

We have carried out a preliminary Phase I trial of cisplatin and DDTC to determine the maximum tolerated dose (MTD) of DDTC when used in combination (9). Cisplatin (50-60 mg/sq m) was infused over 20-30 min and, after a 45-minute delay, DDTC was infused over 45 min. DDTC infusion produced flushing, burning of the mucous membranes, and symptoms of acute anxiety. These effects appeared to be independent of dose and infusion rate, they generally diminished as the infusion proceeded, and there were no serious long-term sequelae. These symptoms were partially alleviated by prior sedation. DDTC doses as high as 7.5 g/sq m have been given without serious adverse effects. Pharmacokinetic studies (Table 2) indicate that DDTC is rapidly cleared and that doses greater than 2.5 mg/sq m give peak plasma and AUC values greater than those required for kidney, gut, and bone marrow protection in rodents (see Table 2 and ref. 4). Prolongation of infusion times beyond 1 hr, however, reduced steady-state plasma concentrations and AUC values below optimum therapeutic levels. We are using a DDTC dose of 4 g/sq m in combination with escalating doses of cisplatin and carboplatin in subsequent clinical trials; the major focus of these studies will be to establish the MTD of the platinum drug with respect to cisplatin neurotoxicity and carboplatin myelosuppression.

Table 2. DDTC pharmacokinetic parameters in man, rat, and mouse

Dose (g/m^2)	$t_{1/2}$ (min)	C_p (uM)	AUC (mM·min)
MAN			
2.8[a]	13.4 ± 0.6	400 ± 36	20.8 ± 1.3
4.6[a]	13.1 ± 0.9	1000 ± 75	55.0 ± 5.8
2.8[a,d]	11.6	305	20.2
2.8[b,d]	10.0	105	8.7
2.8[c,d]	8.8	72	8.0
RAT			
250[e]	10	1170 ± 120	15.2
MOUSE			
250[f]	10	400 ± 50	9.8

[a] iv infusion over 45 minutes.
[b] iv infusion over 85 minutes.
[c] iv infusion over 110 minutes.
[d] same patient.
[e] iv bolus; dose in mg/kg.
[f] ip bolus; dose in mg/kg.

SUMMARY

DDTC reduces most of the major toxicities associated with platinum drug therapy without apparent inhibition of the antitumor effect. The specific mechanisms by which the platinum drugs cause toxicity and DDTC ameliorates these toxic effects are not well understood at present. Although DDTC's protective effects in the kidney may be explained by a chelating mechanism, it is difficult to understand how DDTC might inhibit myelosuppression by this mechanism without reducing antitumor effect. Acute myelosuppression and cumulative neurotoxicity are currently emerging as the major obstacles to widespread use of high-dose platinum drug therapy. DDTC's unique efficacy when used as a

rescue agent may provide a solution to these problems; its clinical potential will be further defined by the results of clinical trials currently underway.

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BIOCHEMICAL MODULATION OF CISPLATIN

S.B. Howell, J. Vick, P.A. Andrews, S. Velury and R. Sanga

INTRODUCTION

New information is rapidly emerging on the kinds of adducts that cisplatin forms with DNA (1-3). However, understanding of the cellular pharmacology of cisplatin, and particularly of how one might alter the cellular pharmacology so as to increase the efficacy of the drug is more limited. We have been interested in finding agents that will alter the activity of cisplatin at the cellular level, and using them as probes to trace the fate of the drug between the time it enters the cell and the time it reacts with DNA. Recently we have found that dipyridamole, a drug used extensively as an anti-coagulant and anti-anginal agent, interacts synergistically with cisplatin to enhance toxicity against both cisplatin-sensitive and resistant human ovarian carcinoma cells.

MATERIALS AND METHODS

Cells lines and Clonogenic Assays.

The human ovarian carcinoma cell line 2008 (4) and a cisplatin-resistant subline (5) were used in these studies. Clonogenic assays were performed by seeding 300 cells per 60 mm plastic dish in the presence or absence of various concentrations of drugs, and counting the colonies that formed after 10 days.

Cisplatin Uptake.

Uptake of cisplatin was quantitated using (^{195m}Pt) cisplatin added to subconfluent cultures of cells. After incubation with the radioactive drug, the plates were extensively washed, the cells were digested with 1 N NaOH, and the gamma emissions counted.

Metabolite Analysis.

Subconfluent cultures of 2008 cells were treated with 100 uM cisplatin for 1 hour. After washing, the trypsinized cell pellet was sonicated and the lysate analyzed by HPLC as described by Daley-Yates and McBrien (6).

RESULTS

Dipyridamole increased the cytotoxicity of cisplatin to both the cisplatin-sensitive and cisplatin-resistant human ovarian carcinoma cells. Using the product of the surviving fractions method (Figure 1) the interaction appeared to be synergistic against both sensitive and resistant cells. Thus, the observed cell kill

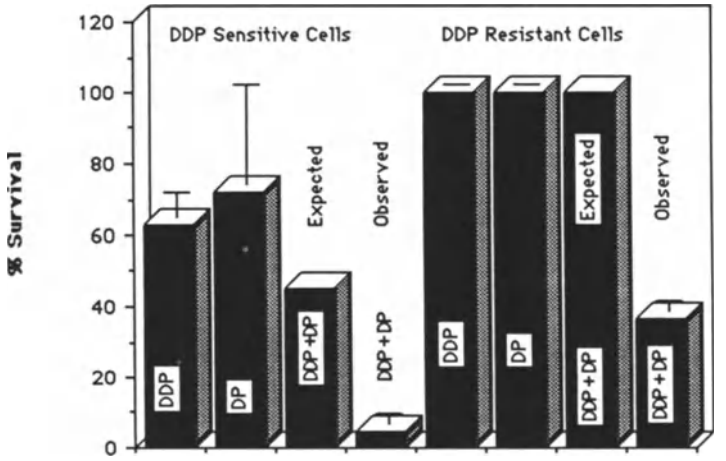

Figure 1. Analysis of the interaction between cisplatin (DDP) and dipyridamole (DP) by the product of the surviving fraction method. Cells were exposed for the full period of colony formation to either 0.2 uM or 20 uM dipyridamole alone or a combination.

was greater than that expected on the basis of the product of the fraction of cells surviving exposure to either cisplatin or dipyridamole alone. A concentration of 20 uM dipyridamole increased the activity of cisplatin by an average of 4.3 ± 1.8 (±SEM, n=3) and 3.6 ± 1.5 (±SEM, n=4)-fold, in the sensitive and resistant cells respectively, when measured as the ratio of the IC_{50}. To confirm

that the interaction was in fact synergistic, isobologram analysis (7) was used to examine the sensitive cells. At the 20% level of cell kill the average interaction index was 0.48 ± 0.07 (± SD, n=3) indicating that only 0.48 times as much drug was required to kill 20% of the sensitive cells as if there was a purely additive interaction between the two drugs. The interaction index at the 50% level cell kill was 0.74 ± 0.03 (± SD, n=3), and at the 80% cell kill it was 0.90 ± 0.06 (± SD, n=3). These results suggested that synergy was more evident at lower levels of cell kill.

The effect of dipyridamole on the uptake of cisplatin into sensitive cells was examined to determine whether this was the basis for synergy. Dipyridamole increased cisplatin uptake to 153 ± 23% (± SD, n=5) when the two drugs were added together. As shown in Figure 2, the uptake was linear over the 60 minutes, and the response to dipyridamole was immediate. An effect of similar magnitude was observed in the resistant cell line as well. Uptake in the resistant cell line in the absence of dipyridamole averaged 67 ± 12% (± SD, n=3) of the uptake in the sensitive cells. The concentration dependence of this effect of dipyridamole was examined in the sensitive cells.

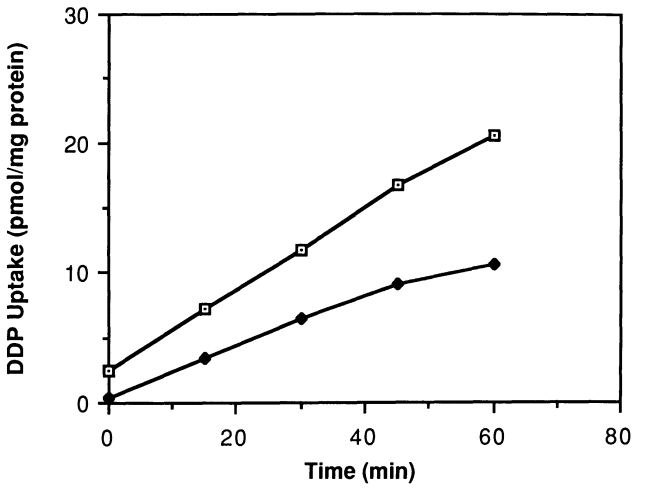

Figure 2. DDP uptake as a function of time in 2008 cells in the presence (□) and absence (◆) of 20 uM DP.

Figure 3 shows that uptake increased rapidly as a function of dipyridamole concentration up to approximately 5 uM, and then less rapidly up to 20 uM.

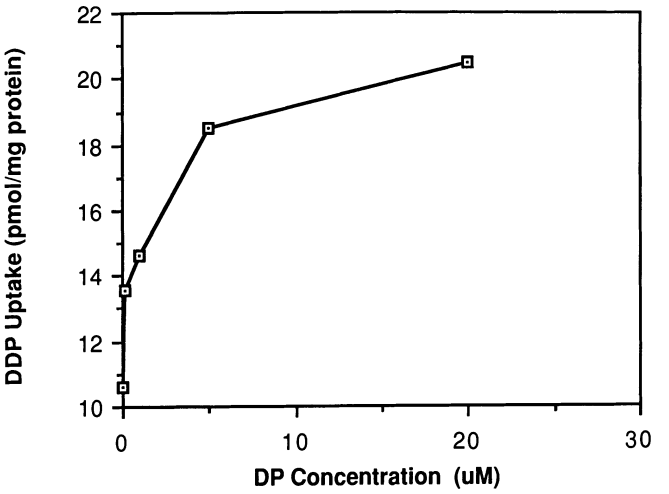

Figure 3. DDP uptake at the end of 1 hour in 2008 cells as a function of DP concentration.

Although the effect of dipyridamole on cisplatin uptake was immediate, when cells were exposed to both drugs for one hour at a dipyridamole concentration of 20 uM, the cytotoxicity was enhanced by only a factor of 1.3 ± 0.2 (± SEM). Pretreatment for up to 4 hours did not demonstrate any greater degree of synergy, but post-treatment with dipyridamole following a one hour exposure to cisplatin, in which the dipyridamole was left in the culture for the full period of colony formation, increased the dose modifying factor to 2.3 ± 0.1 (± SEM).

Dipyridamole is a potent nucleoside membrane transport inhibitor (8). Nitrobenzylthioinosine is also a potent nucleoside membrane transport inhibitor. To determine whether it was via this mechanism that dipyridamole was working, the two drugs were compared for their ability to potentiate the activity of cisplatin. At concentrations which produced comparable decreases in uridine incorporation, the nitrobenzylthioninosine produced a mean dose

modification factor of only 1.2 ± 0.01 (± SD, n=3), whereas dipyridamole produced a dose modifying factor of 4.3 ± 1.8 (± SEM). Thus, it did not appear that the mechanism by which dipyridamole was modulating cisplatin activity was related to nucleoside membrane transport alone.

Seven intracellular metabolites of cisplatin were separable by HPLC using the method published by Daley-Yates and McBrien (6). When sensitive cells are incubated with 100 uM of cisplatin for one hour, in the presence or absence of 100 uM dipyridamole, there was a change in the distribution of the intracellular metabolites (Figure 4). There was a significant decrease in the relative proportion of the native drug and the bis-methionine species, and an increase in all 3 aquated species. Thus, the observed synergy between cisplatin and dipyridamole was associated with an increase in the relative amounts of the most toxic metabolites of cisplatin at the expense of less toxic metabolites.

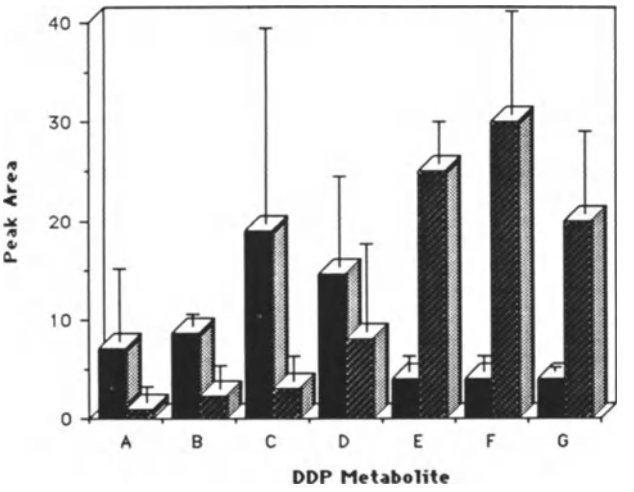

Figure 4. Distribution of DDP metabolites in 2008 cells exposed to 100 uM DDP for 1 hour in the absence (open bars) or presence (hatched bars) of 100 uM DP. Each bar represents the mean of 4 experiments; vertical lines ± SD. A, unknown metabolite; B, native drug; C, bis-methionine complex; D, mono-methionine complex; E,F,G, aquated species.

DISCUSSION

We have demonstrated that dipyridamole interacts synergistically with cisplatin by changing its cellular pharmacology. The synergistic nature of the interaction was established by two independent methods, the product of the surviving product method and isobologram analysis. This effect was observed in both cisplatin-sensitive and cisplatin-resistant cells, and the magnitude of the effect was sufficient to partially restore sensitivity to the resistant cells. There was variation in the degree of synergistic interaction as a function of the fraction of cells killed, with synergy being most pronounced at low levels of cell kill. The synergistic interaction was associated with a substantial increase in the uptake of cisplatin in both the sensitive and resistant cells. It is important to note that one cannot conclude from this result that there was a change in transport of the drug, since a diminution in efflux could equally well account for the result.

In addition to enhancing the uptake of cisplatin, dipyridamole also altered the intracellular distribution of the soluble fraction of the drug. Cisplatin is thought to undergo a complex series of aquation reactions inside the cell, and it is the aquated species which are thought to be most toxic because their ability to react rapidly with DNA. Thus, it is of particular interest that dipyridamole treatment enhances the relative concentration of the aquated species.

Despite the fact that dipyridamole is a potent nucleoside membrane transport inhibitor (8), the inability of nitrobenzylthioinosine, another potent nucleoside membrane transport inhibitor, to influence cisplatin toxicity makes it less likely that it is via this mechanism that dipyridamole is working. Dipyridamole is also an inhibitor of cyclic AMP phosphodiesterase in platelets (9-11), and it may be via changes in cellular cyclic AMP that dipyridamole is mediating its effect.

Although the maximal modulation of cisplatin activity is observed with dipyridamole concentrations which are 2 to 4-fold above those usually achieved in plasma with oral dosing, recent studies have demonstrated that extremely high dipyridamole concentrations are readily achievable when the drug is administered

by the intraperitoneal route (12). Since cisplatin can also be administered by the intraperitoneal route (13), it may be of interest to combine these two agents for the treatment of ovarian carcinoma.

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EFFECTS OF CISPLATIN AND CARBOPLATIN ON NEUROHYPOPHYSIS, PARATHYROID AND THEIR ROLE IN NEPHROTOXICITY

S.K. Aggarwal and J.M. Fadool

INTRODUCTION

Cisplatin (CDDP) is currently one of the most valuable antineoplastic drugs (1) with several toxic side effects of which nephrotoxicity is the major dose limiting factor in its use (2). The primary mechanism of its action has been proposed to be through its cross-linking DNA strands (3). It has also been shown to inactivate various transport enzymes and induce hypocalcemia and hypomagnesemia (4), in addition it causes inhibition of karyokinesis and cytokinesis (5). Cisplatin induces morphological changes in the kidney with hampered urine output depending upon the specie or the strain of animal being used for treatment or testing. Carboplatin (CBDCA) a second generation analogue, however, has proven to be diuretic and less nephrotoxic in some animals. Thus different analogues of platinum have proven to influence the kidney function differently. Cisplatin induces diuresis in Long-Evans rats where as carboplatin is antidiuretic in the same strain of rat (6). Present is an effort to determine the effect of these two drugs on morphological and cytochemical changes in the neurohypophysis and correlate these to any diuretic or antidiuretic influence on the kidney or any hormonal changes in the parathyroid gland that may control the calcium levels in the body.

MATERIALS AND METHODS

Male Wistar rats (Crl:(WI)BR)(Charles River Laboratory, Portage, Michigan) weighing 200 - 300 g were divided into groups of 20 animals and treated with 5 mg/kg, 7 mg/kg, 9 mg/kg of cisplatin in 0.85% saline or 50 mg/kg carboplatin dissolved in 5% glucose solution. Controls received just the injections of vehicle solution. The animals were housed in metabolic cages and provided with food and water ad libitum. Urine output and water intake were monitored for each rat throughout the experiment. Treated and untreated animals were given daily injections (iv) of calcium (0.5 ml of calcium gluconate) and killed on days 3, 5, 10, and 45 post injection. Trunk blood was collected in heparinized tubes. After centrifugation plasma volume was divided by the animals' body weight. The percentage difference between experimental rat blood volumes vs control blood volumes was calculated. Calcium measurements were done on the urine and plasma using flame atomic absorption spectroscopy.

The pituitaries were fixed for 4 hrs in Bouin's solution and processed in a routine manner for paraffin sectioning. Sections (7 μm) were stained with periodic acid-schiff (PAS) techniques before and after salivary amylase digestion and were counter stained with either Orange G or Ehrlich hematoxylin. Chromalum hematoxylin/eosin was used to stain the adjacent sections for the demonstration of neural hormones and pituicyte morphology.

For the immunocytochemical localization of vasopressin, primary antisera, rabbit anti-Arg^8VSP (Biomedia; Forest City, California) was diluted to a final working solution of 1:50. Secondary antisera was goat anti-rabbit IgG, conjugated with alkaline

phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) in a dilution of 1:100. Sections of formaldehyde (4%) fixed pituitaries were incubated in primary antisera for 30 min at room temperature. After proper rinses, sections were incubated in secondary antisera for 30 min. Binding of the labelled antibody was detected by the histochemical demonstration of alkaline phosphatase (6). Endogenous AP activity was inhibited by prior incubation in 0.05 mM Levamisole. Controls included omitting either the primary or secondary antisera. For electron microscopy pituitary and parathyroid tissues were fixed in 1% glutaraldehyde and 1% OsO_4 at 4° C in 0.05 M cacodylate buffer (pH 7.4) for 4 hrs. Thick sections (1 µm) were stained with 1% methylene blue. Ultrathin sections (700 Å) were examined with a Hitachi HU11E electron microscope operated at 75 kv.

To assess the effect of dehydration on the neurohypophysis a group of 20 animals were deprived of water for up to 5 days. These animals were killed on day 1 or day 5 and the pituitaries were processed for light or electron microscopy as described before. The stomachs from the various drug treatments and controls were removed and photographed for bloating or non-bloating effects.

RESULTS

All the cisplatin-treated rats had significant weight loss during the first week of treatment with animals in higher dose groups (7-9 mg/kg) registering the greatest losses. The carboplatin-treated and controls gained weight throughout the duration of the experiment. A striking feature of cisplatin treatment in rats was a marked dose-related decrease in urine output by day 3, paralleled by a significant decrease in water intake

(Fig. 1). By day 5 the urine output continued to decrease with little to no output in 7-9 mg/kg treatment groups while the water intake started to increase, although it still stayed lower than that of the normal animals. Carboplatin-treated rats exhibited an increase in the water intake and urine output 3 days post-injection while recording a drop in both parameters at day 5 (Fig. 1). The water deprived animals excreted negligible amounts of urine on any given experimental day. Morphological and cytochemical studies before and after cisplatin treatment demonstrated the axonal endings within the neurohypophysis lost their characteristic PAS-Chromalum hematoxylin positive granules (Figs. 2 and 3); instead one could find an increase in the number of empty vesicles. Immunocytochemical studies further confirmed the loss of neurosecretory (arginine vasopressin) material from the neurohypophysis after cisplatin-treatment, in a dose dependent fashion. The neurosecretory granules could be resolved better as distinct granules in 1 µm thick sections stained with methylene blue. Under the electron microscope these granules are highly electron dense and seem to arise by the fusion of smaller ones.

Cisplatin-treatment induced some characteristic changes in the pituicytes and axonal endings. Large accumulations of PAS positive material that tested positive for glycogen were observed in the pituicytes (Figure 3). These glycogen accumulations were pronounced after 7 mg/kg cisplatin treatments even after 3 days. At lower dose levels (5 mg/kg) glycogen accumulations do occur but after longer periods (10 days) of treatment. The water deprived animals did show glycogen accumulations in their pituicytes mostly limited to the cortical regions and never in same concentrations as after cisplatin treatments.

Cisplatin treatment or water deprivation, in addition to causing loss of neurosecretory material from the axonal endings, induced a successive rounding up of the pituicytes which was most pronounced after 9 mg/kg treatment and after only 3 days. Such pituicytes lost their cytoplasmic extensions while most of their cytoplasmic organelles showed degeneration. This was characterized by swollen mitochondria, Golgi and the endoplasmic reticulum, and clumping of the nuclear material within the nucleus. Such cells were usually lost after about 10 days, leaving large vacuoles in the gland still observable 45 days post-treatment.

Immunocytochemical demonstration for VSP content of the neurohypophysis using alkaline phosphatase labelled secondary antibody, revealed uniform staining of the neurohypophysis of the normal gland and no staining of the adjacent intermediate lobe of the anterior pituitary. After cisplatin treatment, there was little staining of the neurohypophysis and then only in the cortical regions. This was taken as evidence of a release of the VSP from the neurohypophysis after cisplatin treatment.

In the carboplatin-treated groups after 3 days, the number and size of neurosecretory granules per axonal ending was greater as compared to controls, indicating an increased storage of the hormones. These pituicytes also showed an increase in lipid droplets as compared to the controls. After 5 days the lipid droplets increased, but the amount of neurosecretory material decreased as compared to the 3 day carboplatin treatment group, indicating a release of vasopressin which corresponds well with our urine output data (see Fig. 1).

Cisplatin induced an inactivation of various transport enzymes (ca^{2+}- ATPase, N^{+}/K^{+}- ATPase, alkaline phosphatase and 5'-nucleotidase) from the membranes of the tubular cells of the kidney both in vitro and in

vivo. It also triggered bloating of the stomachs especially in higher dose groups. Daily injections of calcium seem to restore the normal functions of the kidney as measured by the changes in the enzyme levels, the weight lost by the animals and the bloating of the stomachs (Fig. 4). Serum analysis of the cisplatin/calcium injected animals demonstrated normal levels of calcium, however, urine analysis showed fluctuations corresponding with the calcium injections. If animals treated with cisplatin for 3 days were given calcium, there was observed no increase in the serum calcium, instead there was observed a steady decrease. Most of the injected calcium could be recovered in the urine. Fine structural analysis of parathyroid gland from cisplatin treated (3-5 days) animals showed preponderance of active dark cells with highly involuted plasma membranes, extensive endoplasmic reticulum and a nucleus with a highly convoluted nuclear membrane as compared to the normal gland from controls. Calcium injections reversed this trend with predominance of relatively inactive clear cells with little Golgi, endoplasmic reticulum and smooth plasma membranes.

DISCUSSION

The cell lines shown to be susceptible to the toxic drugs usually show destruction of their membrane transport enzymes soon after drug treatment (4). However, cell lines proven to be resistant to various drugs have altered or elevated levels of transport enzymes (7). The latter group may be better able to overcome the destruction of a larger number of enzymes before intracellular ion concentrations are affected and cell function altered. Resistant cell lines of a number of drugs including cisplatin, the cytotoxicity can be enhanced by simultaneous administration of Ca^{2+}-

activated ATPase inhibitors such as verapamil (8), thus lending more evidence to the inactivation of enzymes as a viable action of anti-cancer agents.

Changes in membrane associated transport enzymes will lead to alterations of intracellular electrolyte concentrations and can have an adverse effect upon cell function and proliferation. Cisplatin treatment results in a release of stored Ca^{2+} from mitochondrial fractions (9-10) leading to uncoupling of oxidative phosphorylation. The release of Ca^{2+} from intracellular stores would result in greatly increased cytosolic Ca^{2+} levels. This increase is probably responsible for the depolymerization of microfilaments and microtubules thus arresting cytokinesis and karyokinesis of dividing cells and effects upon cell shape through changes in cytoskeleton structure (4, 5). Nagata and Rasmussen (11) have demonstrated that a rise in calcium levels causes increased gluconeogenesis in isolated kidney tubules. After cisplatin treatment, glycogen accumulations are seen in a number of normal tissues including kidney epithelial cells (9), lymphocytes (12) and endothelial cells in the hypothalamus (unpublished). Low serum calcium levels have been shown to be responsible for the bloating of stomachs in the cisplatin-treated animals (13) indicative of non-contractility of the smooth muscles involved. The stomach smooth muscle strips from such animals have been shown to be hypercontractile to acetylcholine stimuli in a dose related fashion (9) provided these are suspended in low calcium containing medium. Injecting calcium into cisplatin-treated rats prevents bloating of the stomach and possibly restores normal contractibility of the smooth muscle as the peristaltic movement in the intestine can be observed after opening the body cavity. This is not the case in cisplatin-treated animals where the intestines are pale

and show no movement. Cisplatin-treated animals also have been demonstrated to show delayed rigor mortis. Cisplatin seems to induce morphological changes in the parathyroid gland characteristic of various drugs inducing low serum calcium levels (14).

The kidney's vulnerability to cisplatin may stem from its ability to accumulate and retain cisplatin to a greater degree than other organs (15). In an effort to decrease the accumulation of cisplatin in the tubule cells, diuresis therapy has been used in clinical treatments (16). In an effort to improve the therapeutic index for cisplatin, several analogues have been synthesized and tested with changes made at the chloride labile groups or the non-exchangeable amino ligands (1). Though a number of other methods have been used to combat the various toxicities (16), hydration seems to be the method of choice.

Cisplatin has been shown to be diuretic in Sprague-Dawley rats (17) and initially less nephrotoxic as measured by the enzyme levels in the urine when compared to Wistar rats that have little urine output after cisplatin treatment. Immunocytochemical and morphological evidences demonstrate that the change in urine output of the Wistar rats correlate to the release of VSP from the posterior pituitary. There is little to no storage of the pituitary hormones in dense core vesicles and increased incidence of smaller translucent vesicles at the release zones in the axonal endings (18). The release of VSP from the pituitary causes increased resorption of water by the kidney and possible increased accumulation of cisplatin within the cells of such animals leading to increased cytotoxicity.

The morphologic changes of the pituicytes after cisplatin treatment are similar to those of other tissues after cisplatin treatment. Cisplatin's previously

reported antimitotic activity, whether it be through DNA cross-linking (3) or to microtubule depolymerization (5) would account for the lack of replacement of the pycnotic pituicytes. Rounding up of cells, clumping and swelling of the organelles, and glycogen accumulations have all been previously reported in other tissues after cisplatin treatment. These too may be due to cisplatin's effect upon Ca^{2+} levels which play an important role in pituicyte functions (19).

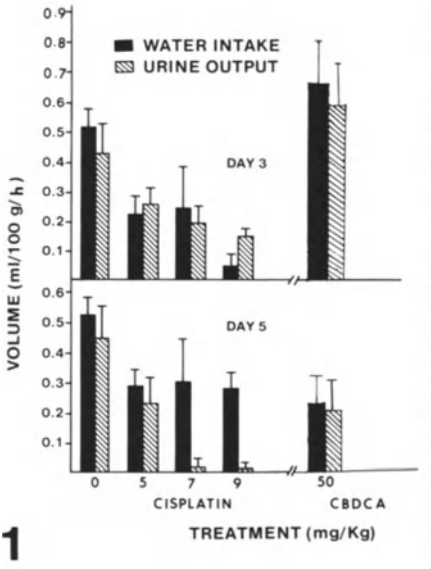
The changes in the pituicytes and subsequent release of neurohormones from the pituitary may also be responsible for the embryo resorptions in rats and mice after cisplatin treatment (20, 21). Much of the evidence given for release of VSP from the pituitary can also be used as evidence of oxytocin (OXY) release as well. The rounding of pituicytes, near total lack of hematoxylin staining axons, and lack of dense core vesicles (18) after cisplatin treatment could only be possible if both VSP and OXY were absent from the pituitaries. Furthermore, after water deprivation both OXY and VSP levels in the circulating blood increase. In pregnant mice and rats, these elevated levels of OXY would cause constriction of the uterine smooth muscle, causing resorption of the implanted embryos thus superseding the changes in hormones responsible for the maintenance of the pregnancy as previously reported (20). Uterine smooth muscle relaxation is under the influence of acetylcholine. If its release is blocked, the smooth muscle would stay contracted and possibly would lead to resorption of any embryos.

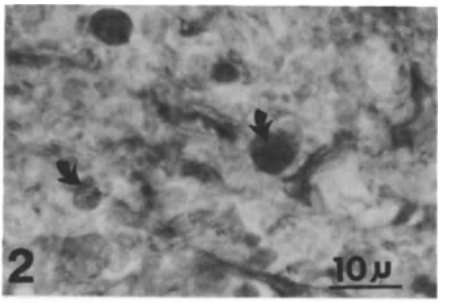
The release of VSP and subsequent decrease in urine output following cisplatin treatment in the Wistar rat differ greatly from those of Sprague-Dawley rats. In the latter strain, cisplatin treatment results in both increased urine output and water consumption with peaks

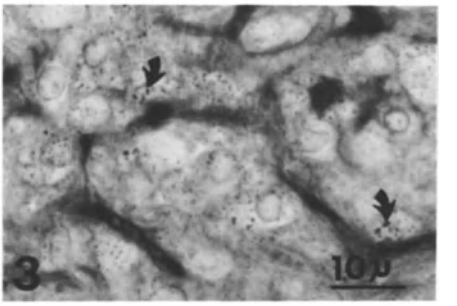
in both parameters first at day 1 and then day 6 post-treatment (22). The first peak was found to be due to lower levels of circulating VSP, but the second peak was due to failure of the kidney. Although the levels of circulating VSP were above normal levels, the kidney response as measured by prostaglandin synthesis was absent (22). The same was true when exogenous VSP was injected indicating possible alterations of hormone receptor sites.

In contrast to the results of the pituitary VSP content, urine output and nephrotoxicity of cisplatin treated Wistar rats are those after carboplatin treatment. Carboplatin treatment results in increased VSP storage through the first 3 days of treatment and a corresponding diuresis. The diuresis could account in part for the much greater excretion rate of carboplatin as compared to cisplatin (15). Up to 90% of the dose of carboplatin is excreted in the urine as compared to only 50% of the cisplatin for the same amount of time. Up through this time there is little associated nephrotoxicity with the carboplatin treatment (6). However, at 5 days post carboplatin treatment, there appears to be a release of VSP from the pituitary and a subsequent decrease in urine output. The lack of water excretion at this time, probably results in increased accumulation of the platinum drug within the kidney, which in turn may be responsible for the rise in lysosomal activity in the tubule cells (6) and a corresponding rise in nephrotoxicity.

In order to evaluate the nephrotoxicity of the platinum coordination complexes, it is important to understand any hormonal influences which may alter the efficacy or toxicity of the drug. It is demonstrated that in the Wistar rat, cisplatin treatment results in release of VSP from the neurohypophysis, increasing water







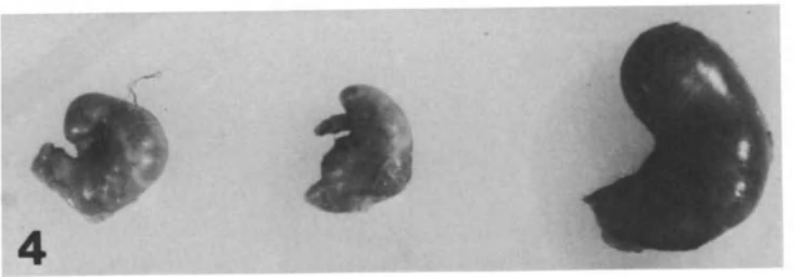


Figure 1: Graph illustrating the 24 hr water intake and urine output volumes (mean±SDM) before and after the cisplatin and carboplatin treatments at day 3 and day 5.

Figure 2: PAS/Orange-G stained 7 µm thick section of a neurohypophysis from a normal rat showing the carbohydrate nature of the neurosecretory material within the axons and the large axonal endings (arrows).

Figure 3: PAS/Orange-G stained section of a neurohypophysis from a cisplatin-treated (7 mg/kg) rat at day 3. Note the large accumulations of glycogen within the pituicytes (arrows).

Figure 4: Comparative stomach appearance of normal, cisplatin and calcium (12.5 mg Ca^{2+}/kg/d) injected, and cisplatin-treated rats.

resorption and possibly platinum re-uptake and accumulation in the kidney.

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CISPLATIN UPTAKE MEDIATED CISPLATIN-RESISTANCE IN HUMAN OVARIAN CARCINOMA CELLS

P.A. Andrews, S.C. Mann, S. Velury and S.B. Howell

INTRODUCTION

Although it has been assumed for many years that DDP accumulates into cells by means of passive diffusion (1), this has been supported only by unconvincing and conflicting reports (2,3). Recently, it has become apparent that decreased DDP uptake is a characteristic of a variety of DDP-resistant cell types. We have reported preliminary studies that two DDP-resistant human ovarian carcinoma cell lines have decreased DDP uptake (4,5). Other workers have also described decreased DDP uptake in DDP-resistant L1210 cells, CHO cells, and SCC-25 human head and neck squamous carcinoma cells (6-11). Characterization of the mechanism(s) of DDP uptake and how it has changed in DDP-resistant cells is clearly an important issue for understanding not only DDP-resistance, but the cellular pharmacokinetics of DDP in general.

METHODS

The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, and a DDP-resistant subline were used in these studies (12,13). All studies were done with mycoplasma negative cells.

DDP uptake studies with [^{195m}Pt]DDP were conducted as described (13). In uptakes conducted with unlabelled DDP, cell

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monolayers were trypsinized instead of digested with NaOH, and the Pt content determined by atomic absorption spectrometry. To assess the effects of osmolarity and pH, DDP uptake experiments were conducted in Hepes buffer consisting of the following: 10 mM Hepes-Tris, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 0.124 M NaCl, pH 7.4

For metabolite analysis, cells were treated with 100 uM DDP in RPMI 1640 media at 37°C for 1 hr. Cells were washed, trypsinized, centrifuged, resuspended in water, sonicated, and then ultrafiltered. The collected ultrafiltrate was injected onto the HPLC column and chromatographed as described (14).

RESULTS

Parent 2008 cells had 2.94 ± 0.40 uL/mg protein (N=4) and 2008/DDP cells had 3.03 ± 0.52 uL/mg protein (N=4) (15). Differences in DDP uptake could therefore not be attributed to changes in cell size. These DDP-resistant cells had been selected three to eight times with 1 uM DDP and were 3 to 4-fold resistant at the time that these uptake studies were conducted.

DDP uptake was linear with concentration from 0.25 uM to 3.1 mM DDP in both parent and DDP-resistant cells (data not shown). The slope of the uptake versus concentration line for 2008/DDP cells was 55.4% of the 2008 cells (9.11 pmol/mg protein/uM). Uptake of 1 uM DDP was linear for approximately 3 hr in both cell lines at which time the uptake began to slow (data not shown). The uptake continued to rise for 24 hr and never showed clear signs of reaching equilibrium. The average uptake for 2008/DDP cells was 59.4 ± 6% of parent cells for the five time points determined.

We found that after one hr of drug exposure, 17.6 ± 4.6% (N=9) of the Pt in the cell lysates was ultrafilterable. The distribution of DDP metabolites that were present in this ultrafiltrate was determined (Fig. 1). All species that were present in parent cells were also present in resistant cells, but in decreased amounts. The average level for the 7 metabolites detected in DDP-resistant cells was 35.8 ± 15.1% of the levels

detected in parent cells. Intact DDP accounted for 37% and 42% of the ultrafilterable DDP in parent and resistant cells respectively.

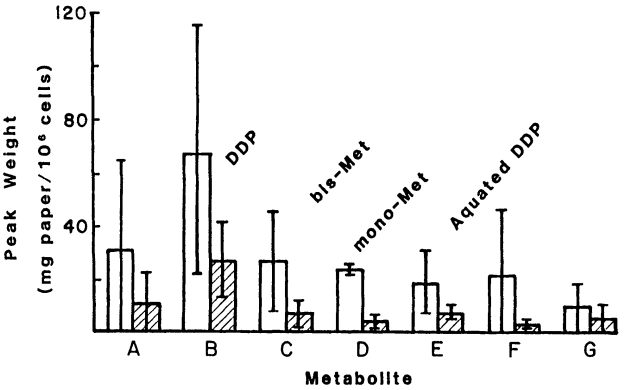

Fig. 1. Distribution of DDP metabolites in parent and DDP-resistant 2008 cells (striped bars). The identity of the peaks are as follows: A, unknown species; B, parent drug; C, bis-methionine complex; D, mono-methionine complex; E, F, and G, aquated species. Bars represent the mean ± the S.D. of three separate experiments.

No reproducible difference in the amount of cell-associated Pt that was available for exodus was observed. After a 1 hr exposure to DDP and then an additional 1 hr in drug-free media, 2008 cells lost 23% of their accumulated Pt compared to 27% for 2008/DDP cells (data not shown).

The effects of various metabolic poisons on DDP uptake were examined in both parental and resistant cells (Table 1). Dinitrophenol and NaF alone did not affect DDP-uptake. Iodoacetate

Table 1
Effect of metabolic inhibitors on cisplatin uptake[1]

Compound	Concentration	% Control Uptake	
		2008	2008/DDP
dinitrophenol	0.25 mM	103.5 ± 4.0	103.7 ± 13.3
NaF	10.0 mM	102.1	93.8
iodoacetate	1.0 mM	129.0 ± 7.7	150.1 ± 49.9
NaF + DNP	-	66.1 ± 11.3	99.4
IAA + DNP	-	42.4 ± 6.6	57.6
ouabain	0.2 mM	76.0 ± 14.7	67.7

[1] Cells were treated with the indicated concentration of inhibitor for 30 min prior to and then 1 hr during exposure to 10 uM DDP. Values are the mean of 2 to 4 separate experiments.

produced a stimulatory affect on DDP uptake. However, when either NaF or iodoacetate was combined with dinitrophenol, DDP uptake was inhibited. An exception was noted with NaF and dinitrophenol in 2008/DDP cells. A 30 min exposure to 0.2 mM ouabain produced a 24.0% decrease in DDP uptake in parent cells and a 32.3% decrease in 2008/DDP cells. This decrease becomes 50% when cells are pre-incubated 1 hr instead of 30 min. The decrease was dependent on ouabain concentration between 0 and 0.5 uM, no further decrease occurred up to 0.2 mM (data not shown).

DDP uptake was markedly dependent on osmolarity (Fig. 2). Uptake in 2008 cells increased 15.2-fold between 390 and 227 mOsmol/L. DDP uptake in 2008/DDP cells increased 8.8-fold over the same range of osmotic strength. DDP uptake was pH dependent but showed no optimum between pH 6 and 8 (Fig. 3). Resistant cells showed less of a pH dependence than parent cells.

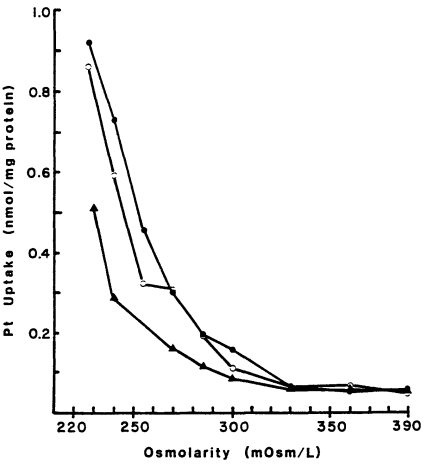

Fig. 2. DDP uptake vs. osmolarity. ●, ▲ NaCl; o, mannitol; ●, o 2008 cells; ▲, 2008/DDP cells.

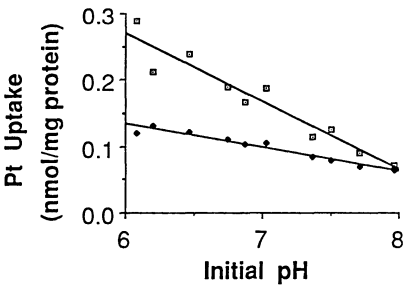

Fig. 3. DDP uptake vs. pH. ⊡, 2008 cells; ◆, 2008/DDP cells.

DDP uptake could not be inhibited with structural analogs (Table 2). Carboplatin in 60-fold excess had no effect on DDP uptake. trans-DDP appeared to stimulate uptake. Most significantly, cis-diamminedichloropalladium(II) only minimally inhibited DDP uptake. This compound is virtually identical to DDP

structurally and if a carrier protein was involved should have decreased DDP uptake by 70%. The 20% decrease observed may relate to damage from non-specific palladination since the palladium analog is some 10^5 more reactive than DDP.

Table 2

Effect of structural analogs on DDP uptake

Analog	Concentration	% Control Uptake		
		2008	2008/DDP	(N)
carboplatin	600 uM	104.7%	96.3%	(2)
trans DDP	18 uM	139.0	132.0	(2)
cis-$PdCl_2(NH_3)_2$	24 uM	78.6 ± 4.7	83.1 ± 12.1	(3)

Values are the mean of two or three separate experiments conducted with duplicate wells. DDP concentration was 10 uM.

The alteration in DDP uptake in resistant cells and the lack of any evidence of carrier-mediated transport suggested that these cells had a change in the physico-chemical properties of their membranes. Analysis of whole cell lipid extracts, however, did not demonstrate a change in total phopholipids, the distribution of phospholipids, or cholesterol content in 2008/DDP cells (data not shown). Fluorescence polarization studies on whole cells at 37°C with trimethylammonium-diphenylhexatriene as a probe indicated that the membranes of resistant cells were less fluid (16). Parent cells had P = 0.320 ± 0.019 (N=4) and resistant cells had P = 0.368 ± 0.020 (N=4, $p < 0.05$).

DISCUSSION

Numerous studies are beginning to appear that identify decreased uptake of DDP as a factor in the in vitro acquisition of resistance to DDP (4-11). We have found that in our 2008 human ovarian carcinoma cells that decreased DDP uptake is expressed after only 3 selections with DDP. The 50% decrease in uptake can account for the majority of the 3 to 4-fold resistance developed to this point. Although the specific biochemical change that accounts for decreased DDP uptake has not yet been identified, we have found that DDP uptake in resistant cells was less affected by osmotic changes, less affected by pH changes, and that the membranes of resistant cells are less fluid than parent cells. Other reports

are now appearing that show that certain compounds (e.g. ouabain, anguidine (17), dipyridamole (18), and aldehydes (19)) can modulate DDP-uptake. These findings hold promise that pharmacologically useful means will be found for circumventing DDP uptake mediated DDP-resistance.

At one hr we found that approximately 7% of the cell-associated Pt was intact DDP. If the assumption is made that this percentage does not change with time or DDP concentration, then one can calculate that although the intracellular concentration of total Pt surpasses the extracellular concentration of 1 uM DDP at 20 min for parent cells and 35 min for DDP-resistant cells, the concentration of intact DDP does not reach 1 uM until 10 hr for parent cells and 28 hr for DDP-resistant cells. This data thus provides compelling, although not definitive, evidence that DDP is not transported uphill against a concentration gradient and that primary active transport is not inolved in DDP uptake.

Combinations of dinitrophenol, an inhibitor of oxidative phosphorylation, and NaF or iodoacetate, inhibitors of glycolysis, decreased uptake approximately 50%. These observations have also been made in L1210 cells (9). We found an equivalent inhibition of DDP uptake with ouabain, a specific inhibitor of Na,K-ATPase. This suggests that the inhibition of uptake by ATP starvation may be similarly mediated by incapacitating the Na,K-ATPase. The inhibition of uptake by ouabain indicates that either DDP uptake is dependent upon the electrochemical gradient across plama membranes or, less likely, that DDP is transported by the Na,K-ATPase. It is interesting to speculate that the amount of Na,K-ATPase in a cell may somehow be a determinant of how much DDP is taken up; the primary targets of DDP toxicity, i.e. the kidney, inner ear, and peripheral nerves, are all tissues that are rich in Na,K-ATPase.

No evidence can be provided that transport of DDP is carrier mediated. DDP uptake can not be saturated, does not appear to be concentrative, does not have a pH optimum, and cannot be inhibited with structural analogs. Other evidence that would indicate that DDP uptake is carrier mediated such as dependance on co-transport or competitive exchange, have not as yet been reported. We are

thus left with the paradox that DDP uptake is not carrier mediated, but is partially energy dependent and ouabain inhibitable. This paradox may possibly be explained by pinocytosis; however, the extent to which pinocytosis contributes to DDP uptake is not yet known.

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INTEGRATED POLYMERASE AND EXONUCLEASE ACTIVITIES OF *ESCHERICHIA COLI* DNA POLYMERASE *I* ON PLATINATED DNA

F. Bernges and E. Holler

INTRODUCTION

Escherichia coli DNA polymerase I was chosen as a model for integrated enzymatic DNA polymerization and excision repair system acting on DNA that was coordinated to cis-diammineplatinum(II) (cis-DDP) or trans-diammineplatinum(II) (trans-DDP), respectively. The protein inherent DNA polymerization, 3`-5`-exonuclease and 5`-3`-exonuclease activities have been measured following incorporation or excision of radioactively labelled nucleotides in/from platinated DNA. The activities were measured as a function of kinetics of DNA platination. Correlation of observed inhibition with previously established kinetics of platination of DNA (1) revealed the stage at which platinum adducts became inhibitory, in particularly whether they were of the monofunctional or crosslinking type.

MATERIALS AND METHODS

Cis-DDP and trans-DDP were gifts from Degussa,

Homburg, FRG. Salmon testes DNA and EDTA were purchased from Sigma, radioactive compounds were from Amersham, glycine and DNA from herring sperm were obtained from Serva, Escherichia coli DNA polymerase I was prepared according to (2). Phage fd 109 infected E.coli was a gift from Dr.W.Oertel (Regensburg). All other compounds were obtained from Boehringer or Merck.

Cis-DDP (trans-DDP) was dissolved at 60 °C for 1 hour (15 hours) in a solution of (10mM KNO_3, pH=5) in the dark to give a final concentration of 2 mM (0.5 mM).

Cis-diammineaquachloroplatinum(II) (cis-aquCl), cis-diamminediaquaplatinum(II) (cis-aqu_2) and trans-diammineaquachloroplatinum(II) (trans-aquCl) were prepared by the $AgNO_3$ method (3). Solutions of the aquated species were prepared freshly for each experiment. All kinetic measurements were evaluated as described (1).

Inhibition of DNA synthesis

The reaction mixtures (4 ml) usually contained 0.4 mM (with regard to nucleotides) activated salmon testes DNA and 0.02 mM platinum(II) compound to give a final r_b=0.05 (rb is the abbreviation used for mol Pt/mol bases of DNA) in (10 mM KNO_3, pH=4.8-5.3). The reaction was carried out at 37 °C in the dark. At different time intervals aliquots (100 µl) were added to 20 µl of quenching mixture (495 mM glycine/NaOH, pH=9.2, 45 mM $MgCl_2$, 0.75 mM EDTA, 37.5mM NaCN) at 0 °C and subsequently kept on ice for 30 minutes. 25 µl of dNTP-mixture (200 µM each of dGTP, dCTP, dATP, and 20 µM of [^{3}H]dTTP (1 Ci/mmol)) and 5 µl of enzyme solution (0.03 units Pol I, dissolved in (50 mM potassium phosphate buffer, pH=7.0, 0.025 mM EDTA, 50 % glycerol)) were

added and the reaction mixture (150 µl) was incubated at 37 °C for 15 minutes. The reaction was stopped and radioactivity counted according to (1). Michaelis-Menten parameters were determined at pH=7.5 under assay conditions (in the absence of CN^-) as described (1).

Inhibition of 3`-5`-exonuclease

Preparation of 3`-endlabelled salmon testes DNA was performed using standard procedures and E.coli Pol I as enzyme. Different preparations were made using either [$^3$H]-labelled dGTP, dATP, dTTP, or dCTP respectively and the 3 corresponding unlabelled deoxyribonucleoside 5`-triphosphates (dNTP`s).

For platination the reaction mixtures (10 mM KNO_3, pH=5, final volume 112,5 µl) contained 0.2-1.6 mM 3`-endlabelled [^{3}H]DNA ($1 \cdot 10^5$ dpm/nmol bases of DNA) and platinum (II) compound to give a final rb=0.1.

The platination reaction was carried out at 37 °C. Aliquots (2 µl) of the reaction mixture were drawn and pipetted into 143 µl of quenching mixture (69 mM glycine/NaOH, pH=9.2, 6.9 mM $MgCl_2$, 0.1 mM EDTA, 2.1 mM β-Me, 3.46 mM KNO_3, 5,2 mM NaCN). The mixture was kept on ice for 30 minutes, 0.1-0.2 units Pol I (5 µl of solution as above) were added and the reaction was allowed to proceed for 90 minutes at 37 °C. After addition of 100 µl carrier mixture (0.5 g/100 ml DNA from herring sperm, 50 mM $Na_2P_4O_7$, 50 mM EDTA) DNA was precipitated with trichloroacetic acid. The precipitate was removed by filtration on GF/C filters and the filtrate counted for radioactivity (1).

Inhibition of 5`-3`-exonuclease

For preparation of template Phage fd 109 double stranded DNA (ds-DNA) and single stranded DNA (ss-DNA) were isolated from cultures of infected E.coli using standard techniques.

The ds-DNA was cut with the restriction enzyme HpaII and the resulting fragments were annealed to ss-DNA. One replication circle was performed using Klenow-Fragment of E.coli Pol I and α-[^{32}P]dATP (100 Ci/mmol). The product was again cut with HpaII, annealed to ss-DNA and again one replication circle was performed using unlabelled dNTP's for elongation of the [^{32}P]dATP-labelled primer fragments.

Based on the specific activity of [^{32}P]dATP and the amount of DNA that was employed the specific activity of the product was calculated 1400 dpm/pmol bases of DNA at a concentration of 4.4 µM bases of DNA.

After removal of Tris-buffer by dialysis the platination was carried out at 20 °C in a solution of (10 mM KNO_3, pH=5) using rb=0.1 or rb=0.05 and 15 to 100 hours for reaction time.

The 5`-3`-exonuclease assay was performed in (50 mM Tris/HCl, pH=7.5, 100 mM NaCl, 7 mM $MgCl_2$, 2 mM KNO_3, 1 mM β-Me, 50 µM each of dGTP, dATP, dTTP and dCTP, 2.5 units/ml E.coli Pol I and 57 pmol bases of DNA) at 37 °C in a final volume of 100 µl.

At different time intervals aliquots (5 µl) were drawn and pipetted into 95 µl of carrier mixture (see above). DNA was precipitated by addition of 100 µl 2 M trichloroacetic acid in an Eppendorff microfuge. 100 µl of supernatant were counted for radioactivity.

Nitrocellulose filter binding assay

Platination of [^{3}H]-endlabelled salmon testes DNA was performed over night at 20 °C in (10 mM KNO_3, pH=5) using concentrations of 1.3 mM in case of DNA and 0.13 mM in case of platinum compound.

The typical reaction solution contained 3.9 nmol of [^{3}H]dTTP-labelled DNA (25.000 dpm/nmol bases of DNA) and E.coli Pol I ranging from 1.3-30 pmol (based on the protein concentration of the enzyme preparation) in a final volume of 50 µl (20 mM potassium phosphate, pH=7.4, 0.8 mM EDTA, 0.025 mM DTE).

For measurement of platinum-induced structural changes of DNA E.coli Pol I was omitted.

The samples were placed on ice for approx. 30 minutes, filtered through nitrocellulose membranes (Schleicher & Schüll, Dassel, FRG) and washed once with 3 ml of cold potassium phosphate buffer (see above). Radioactivity on the membranes was counted by liquid scintillation.

RESULTS

DNA polymerization

The K_M values of the dNTP`s were not affected by platination of DNA at varying rb from rb=0 to rb=0.2. The Michaelis-Menten parameters for platinated DNA as the varied substrate reflected inhibition of DNA synthesis due to a 4-fold increase in K_M in the range below rb=0.1 and an abrupt manyfold decrease in V_{max} in the range of rb=0.06 to rb=0.1 in the case of cis-DDP. In case of trans-DDP, V_{max} decreased over the whole range of rb, and K_M of platinated DNA increased above

rb=0.1 (Fig. 1, 2).

The kinetic analysis of the platination reaction was followed as the degree of inhibition of Pol I dependent DNA-synthesis as described (1).

Table I. Kinetics of platination of DNA measured as inhibition of E.coli Pol I catalyzed DNA synthesis :

compound	k_{2nd} ($M^{-1}min^{-1}$)	k_{obs} (min^{-1})	
cis-(aquCl)	200 ± 30	0.02 ± 0.01	
cis-(aqu_2)	1300 ± 300	0.03 ± 0.01	
trans-(aquCl)	300	0.01	*)

*) = single experiment at 20 °C

3`-5`-(Proofreading-)nuclease

Experiments showed a decreased level of excision of nucleotides via 3`-5`-exonuclease activity as a function of rb. This dependence was linear ranging from rb=0 to rb=0.2, showing 50 % reduced level at rb=0.1 with regard to excision in the absence of platinum for DNA labelled with [^{3}H]dATP and [^{3}H]dTTP, 40 % for labeling with [^{3}H]dCTP and 70 % for [^{3}H]dGTP, respectively.

Platination was performed of [^{3}H]dGTP-labelled DNA and the extend of platination was measured as the level of nucleotides resistant against 3`-5`-nuclease excision. Observed rate constants are summarized in Table II.

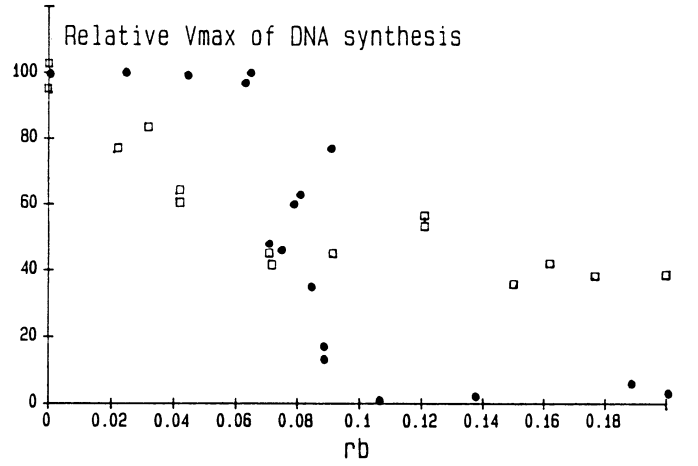

Fig.1: Effect of platination of DNA on the relative V_{max} of DNA-synthesis.
● = cis-DDP, □ = trans-DDP

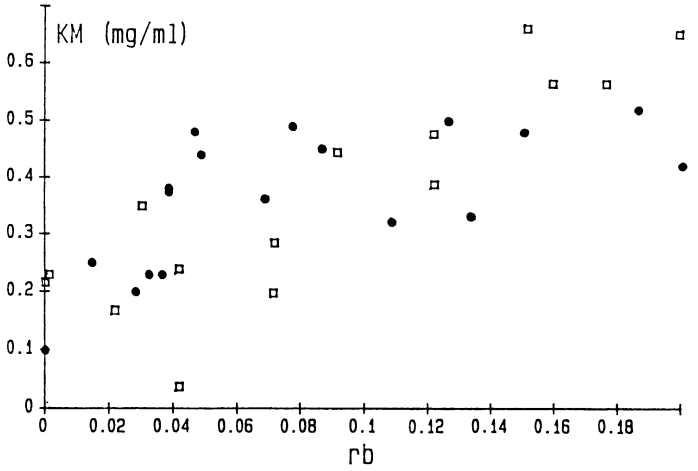

Fig.2: Effect of platination of DNA on the K_M-value of DNA.
● = cis-DDP, □ = trans-DDP

Table II. Rate constants of DNA platination measured via the level of the excision-resistant 3`-[^{3}H]endlabelled nucleotide. Biphasic kinetics were evaluated as described (1). Relative concentrations were rb=0.1.

platinum(II) compound	radioactive nucleotide observed	rate constants first order (min^{-1})	second order ($M^{-1}min^{-1}$)
cis-(aquCl)	[^{3}H]dGMP	0.01 ± 0.003	-
trans-(aquCL)	[^{3}H]dGMP	0.007	- *)
cis-(aqu_2)	[^{3}H]dGMP	0.03	900 *)
cis-(aqu_2)	[^{3}H]dCMP	0.01 ± 0.003	900 ± 200

*) = single experiment

5`-3`-(Repair-)nuclease

The time dependence of platination measured in terms of inhibition of 5`-3`-exonuclease agreed well with that calculated on the basis of established second order rate constants (1), 150 $M^{-1}min^{-1}$ in case of cis-DDP and 300 $M^{-1}min^{-1}$ in case of trans-DDP. This is in agreement with the assumption that the bimolecular attack of DNA by the platinum(II) compound was rate-limiting for the conditions of DNA concentration used (4.4 μM)

By comparison of the number of 5`-3`-excisions and the lenght of HpaII restriction fragments, it was calculated that platination caused termination of excision

on 1 adduct out of 40 adducts excised in the case of trans-DDP and 1 adduct out of 8 adducts excised in the case of cis-DDP.

Nitrocellulose filter binding assay

As shown in Figure 3, upon platination of DNA (rb=0.1) a decrease of the binding capacity of E.coli Pol I towards DNA was observed. In case of rb=0 the value corresponding to half maximum binding was measured 3 pmol E.coli Pol I, which in this case is equivalent with the retention of 300 bases of DNA per molecule of E.coli Pol I. Upon platination of DNA this value is reduced by a factor of 3, equivalent to 100 bases of DNA per molecule of E.coli Pol I.

The filter assay also showed structural changes of the DNA in the absence of E.coli Pol I that occured upon platination (Fig. 4). Because single stranded DNA is retained on nitrocellulose filters, while double stranded DNA can pass through the membrane under the conditions employed, those structural changes can be explained by platinum(II)-induced formation of single stranded DNA.

DISCUSSION

The Michaelis-Menten parameters of platinated DNA in combination with the retention of DNA on nitrocellulose filters reflected structural changes of DNA occuring upon platination. Especially the abrupt fall in V_{max} for cis-DDP correlated with retention of platinated DNA on nitrocellulose filters probably due to melting of DNA around positions of platinum crosslinks.

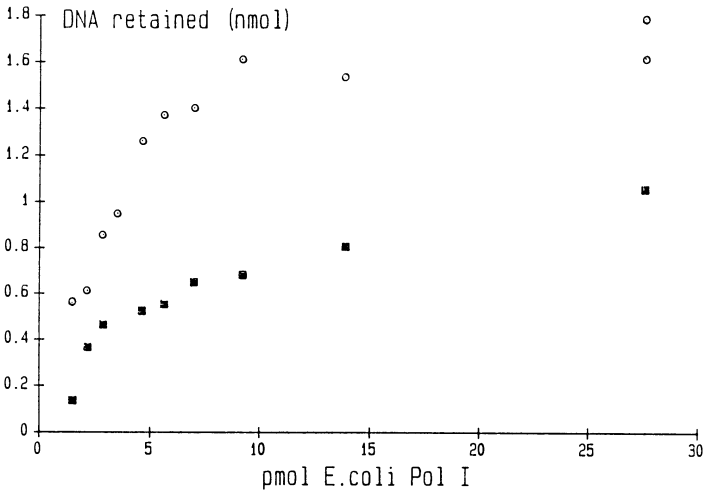

Fig.3: Nitrocellulose filter assay for binding of E.coli Pol I with DNA, rb=0 (o) and rb=0.1 (■).

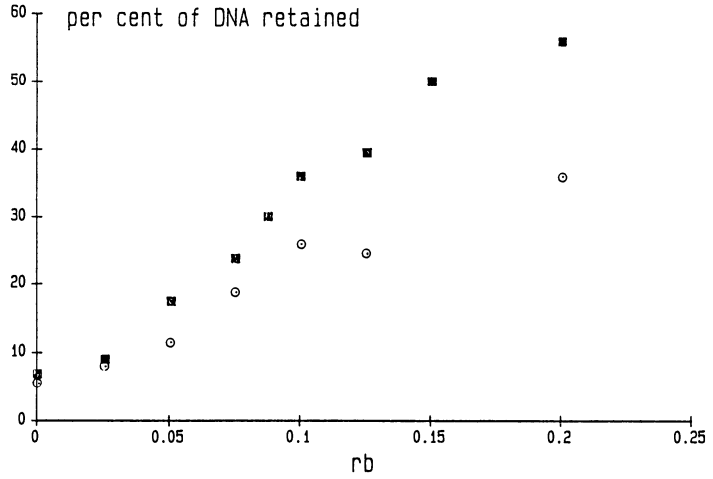

Fig.4: Nitrocellulose filter assay for measurement of structural changes caused by platination for trans-DDP (■) and cis-DDP (o).

The kinetic analysis of the platination reaction indicated adducts of monofunctional type to be responsible for the increase in the DNA K_M-value and crosslinking followed by concomittant melting responsible for decrease in V_{max}.

This mechanism of platinum binding to DNA was supported by the results obtained from the kinetics of inhibition of E.coli Pol I 3`-5`-exonuclease activity. The observed kinetics in case of diammineaquachloroplatinum(II) was consistent with the hydrolysis of the second chloro ligand to be the rate limiting step and slower than crosslinking. Results supported the mechanism presented before (1).

In case of diamminediaquaplatinum(II) the inhibition of 3`-5`-excision of nucleotides following bimolecular platination kinetics (900 $M^{-1}min^{-1}$) could be the result of the fast "conformational rearrangement" (1, 5) that could have occured after the addition of glycine buffer.

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PRELIMINARY RESULTS ADDED AS A NOTE

The mechanism of the platination reaction reported above requires monofunctional adducts for intermediates. Adducts of that kind have now been observed using HPLC separation of the digestion products of DNA according to (4).

After total digestion of [^{3}H]dGTP-labelled DNA using E.coli Pol I or Klenow Fragment 3`-5`-exonuclease activity the products (platinated and unplatinated deoxyribonucleoside 5`-monophosphates) were separated on a C_{18} reversed phase column (LKB, TSK ODS-120T, 5 µm, 4.6 x 250 mm). UV and [^{3}H] traces were monitored continuously and peaks were identified acording to their migration behaviour with reference to standards.

ADMINISTRATION ROUTE OF *CIS*-PLATINUM PLAYS AN IMPORTANT ROLE IN THE *IN VIVO* PHARMACOKINETICS

P. Arslan, P.P. Cagol, P.P. Da Pian, P.L. Pilati, P. Gori and M. Beltrame

INTRODUCTION

For the maximum potential of an antineoplastic drug to be realized, a torough understanding of its pharmacology "in vivo" is essential. The investigation, therefore, on the tissue distribution of Platinum are interesting both from a pathophysiology point of view, as they give an insight into the mechanism leading to different tissue concentrations of this drug, and from a clinical point of view, since they provide a pharmocological explanation for organ-specific toxicity or for antitumor activity.

Generally clinical studies in this field show that cis-Pt, after sistemic administration is concentrated in the animals in the kidney, liver, muscle and skin and in the man, also in ovary, testis, uterus and fat (1,2). In addition, tumor platinum tissue concentration varied with the site of origin of the tumor, with the site of the metastases, if present, and with the route of cis-Pt administration (3).

The study described in this paper involved cis-Pt measurements in liver tumor metastases, using rats in which metastases were induced by injection through portal vein of tumor cell line Walker 256 carcinoma. We have measured, as low as ng/mg of liver dry tissue the amount of cis-Pt retained by liver metastases, by the surrounding healthy liver parenchima and by the kidney. Cis-Platinum was alternatively administered in rats by sistemic route, by portal vein or by hepatic artery.

The amount of cis-Platinum was measured by a very sensitive method, in which wet tissue was first digested and then the platinum was determined as atomic platinum. The aim of the present investigation is to assess the validity of the choice in clinical practice between the loco-regional and the sistemic drug administration, when chemotherapy is

used in non operable liver tumor metastases. The loco-regional treatment is actually evaluated the best way to achieve the highest drug concentration, with the lowest sistemic toxicity.

We have attempted to study a significant correlation between cis-Pt concentration and size of metastases. Under our experimental conditions, between small-size metastases (1-5mm) and medium-size metastases (5-10 mm), cis-Pt uptake and metastasis diameters follow a direct relationship when the drug is administered by hepatic artery. Above 5 mm metastasis diameters, data already reported in literature (4,5) pointed out that between blood flow and metastasis diameters there is an inverse relationship which explains the reduced uptake of cis-Platinum by large-size metastases (above 10 mm) when compared with the drug uptake by medium-size metastases (5-10 mm).

METHODS AND MATERIALS

1. Protocol of cis-Pt administration

Albino Wistar rats, in which liver metastases were induced by injection through the portal vein of cells of a tumor line, Walker 256 carcinoma, were treated with Cis-Pt, after the appearance within 10 days of metastases.

The route of administration of the drug were alternatively, the systemic route, the portal vein or the hepatic artery, in a dose varying from 0.3 mg/100 g of rat body weight if the drug was injected through the systemic route and 0.15 mg/100 g of rat body weight if the route of administration was the portal vein or the hepatic artery.

The maximum diameter of the metastases was measured after the excision of the metastases from the surrounding normal liver and the metastases were divided in small (from 1 to 5 mm), medium (from 5 to 10 mm) and large-size (more than 10 mm).

2. Measurements of platinum tissue contents

Samples of tumor tissue, of the surrounding liver parenchima and of the kidney were removed by a biopsy after the Cis-Pt administration. The tissue samples were weighed, dried and digested in nitric acid (2 hours at 160° C). After the nitric acid evaporation and the following dilution with water, Platinum was measured by flameless atomic absorption spectrophotometry apparatus (Perkin-Elmer 1030), equipped with graphite microoven (6).

For each experiment, a standard curve of Platinum was performed and the controls on healthy liver and kidney were run in parallel.

The data are given in ng/mg of dry tissue.

All chemical used were of analytical grade and commercially available. The pharmaceutical name of cis-Pt was Platinex, Bristol, U.K.

TYPICAL STANDARD CURVE FOR PLATINUM ANALYSIS BY FLAMELESS ATOMIC ABSORPTION SPECTROMETRY.

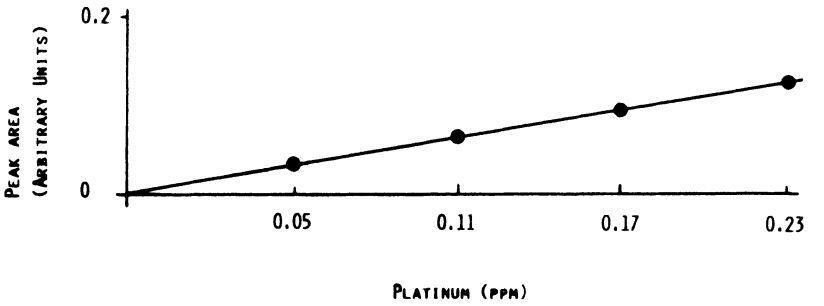

Fig.1. Typical standard curve of platinum analysis by flameless atomic absorption spectrometry apparatus (Perkin Elmer 1030) equipped with graphite microoven.

RESULTS

Table I shows the Cis-Pt concentrations in rat liver metastases and in the surrounding liver parenchima, expressed as ng/mg of dry tissue.

Based on our data, we can emphasize that the arterial administration route allows to get the highest Platinum concentration in the metastases, in view that liver metastases take up from 4 to 7 times more Cis-Pt than the surrounding liver tissue, while in the systemic and portal administration the amount of Cis-Pt retained by tumor and liver parenchima is approximately the same.

Analysis of the diameter of metastases revealed a possible correlation between the size of metastases and Cis-Pt concentration, since the drug injection by hepatic artery may have resulted in higher concentration of drug in metastases, their diameter ranging from 5 to 10 mm.

Table II shows the Cis-Pt concentration in rat kidney, expressed as ng/mg of dry tissue, in relation to the route of Platinum administration. From these values, it is possible to calculate that drug concentration in the kidney is the lowest, as Cis-Pt is injected through the hepatic artery.

TABLE I. Cis-Pt concentration in rat liver metastases and in the surrounding liver parenchima in ng · mg^{-1} dry tissue.

Liver Metastases Diameters	Sistemic Administration	Portal Vein Administration	Hepatic Artery Administration
		ng · mg^{-1} dry tissue	
Ø 1-5 mm	6.7(6)	11.8(6)	20.0(6)
Ø 5-10 mm	9.0(6)	9.7(6)	37.5(6)
Ø 10 mm	8.2(6)	8.1(6)	35.6(6)
Surrounding Liver Parenchima	9.7(6)	8.3(6)	5.4(6)

In parenthesis number of experiments.

Liver metastases were obtained by injecting through portal vein cells of a tumor line, walker 256 carcinoma, in Albino-Wistar rats.
Metastases were isolated from the liver and the maximum diameter was measured in mm. For determination, the metastases of the same diameter were pooled together.
Cis-Pt was administered alternatively by the systemic route, by the portal vein and by hepatic artery.
Cis-Pt was determined by atomic absorption, after digestion of wet tissue.
Standard deviation never exceeded ± 5%.

TABLE II. Cis-Pt concentration in rat kidney in ng · mg^{-1} dry tissue.

	Sistemic Administration	Portal Vein Administration	Hepatic Artery Administration
		ng · mg^{-1} dry tissue	
Rat Kidney	69.4(4)	57.2(4)	27.1(4)

In parenthesis number of experiments.

Cis-Pt was administered alternatively by the systemic route, by the portal vein and by hepatic artery.
Cis-Pt was determined by atomic absorption, after digestion of wet tissue.
Standard deviation never exceeded ± 5%.

Figure 2 shows that the concentration ratio of Cis-Pt in metastases and surrounding liver parenchima is apparently a function of diameter of liver metastases. Using the systemic or portal vein administration, the Cis-Pt concentrations measured in metastases and in normal surrounding liver are not significantly different and the concentration ratio is equivalent to unity and independent of size of metastases. A ratio exceeding unity was found when we used Cis-Pt by arterial route.

ADMINISTRATION ROUTE INFLUENCES THE RATIO BETWEEN LIVER METASTASES PLATINUM CONCENTRATION AND SURROUNDING HEALTHY LIVER PLATINUM CONCENTRATION

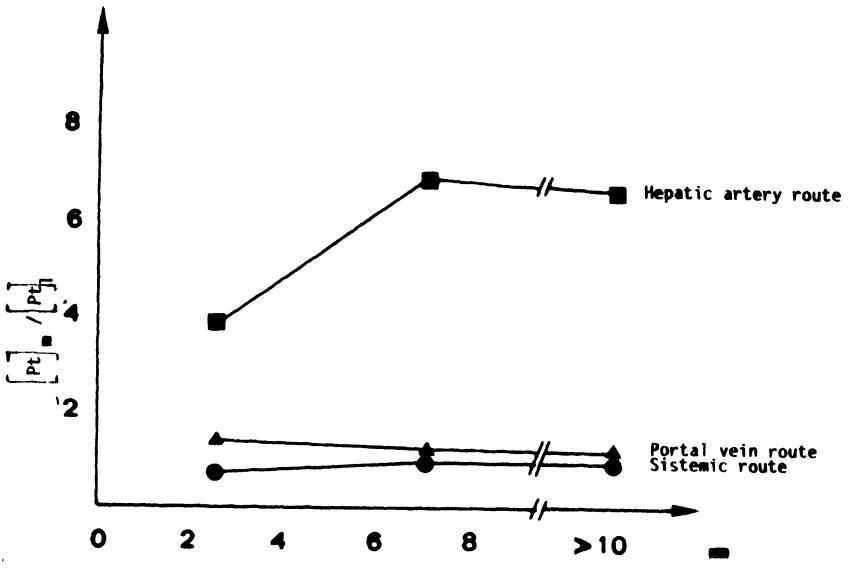

Fig.2; Correlation between $[Pt]_m / [Pt]_l$ ratio and average values of diameters of liver metastases. The platinum determination was assessed by flameless atomic absorption. Details in " Methods and Materials" section. Each point is a mean of 6 different experiments. Standard errors never exceeded ± 5%

$[Pt]_m$ = cis-Pt concentration in liver metastases

$[Pt]_l$ = cis-Pt concentration in normal liver parenchima

DISCUSSION

It has been previously pointed out (7,8,9) that intrahepatic tumor derives most of its blood supply from the hepatic artery, whereas the normal liver derives 80% of its blood supply from the portal venous system, and that the arterial administration of Cis-Pt has been shown to be highly effective in the treatment of localized malignancies (10).

Our data emphasize that the route of administration of Cis-Pt is very important, since the local tissue uptake of this drug is enhanced by intraarterial injection.

Therefore, there are two possible rationales for the use of the arterial route for administration of chemotherapy. The first is that the arterial delivery may result in greater total drug exposure for the metastases. The second is that the drug retained and concentrated in the tumor may decrease the amount of drug reaching the venous circulation, and thus resulting in a decrease of the systemic toxicity, as it is possible to evaluate by Cis-Pt concentrations in rat kidney.

Many authors reported (4,11,12) that the behaviour of blood flow varies according to the size of the tumor, showing an inverse relationship with its diameter; in particular, small-size metastases showed blood flow similar to or slightly higher than that in liver, while large ones were distinctly hypoperfused or showed a quite stagnant blood flow (4).

In addition, Da Pian et al. (5) reported an increase in blood flow in small-size metastases, when they used an intraarterial perfusion, but a decrease in the measurement of blood flow, using the portal or systemic routes.

Our results are in good agreement with data reported in the literature, since Cis-Pt is concentrated in metastases 7 times more than in the part of liver free of tumor, when the drug is injected by intraarterial route and the diameter of metastases ranges from 5 to 10 mm. The relevance of these findings is due to the fact that the local drug concentration shows an evident relationship with metastasis vascularization. The decrease in blood flow in large-size metastases and, consequently, in the Cis-Pt concentration might be due to the distortion and compression of intrahepatic vessels (13), with the consequence that areas of necrosis are present.

However, there is little pharmacokinetics data to support the rationale for intraarterial infusion of Cis-Pt and pharmacokinetics measurements reported have depended on the measurement of total Platinum ion rather than active Cis-Pt. The measurement of bound and free Platinum and the assessment of active and non-active Platinum is absolutely necessary to evaluate the efficacy of loco-regional chemotherapy.

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CYTOTOXICITY OF THE DIETHYLDITHIOCARBAMATE ANION, OF ITS S-METHYLESTER AND OF RESPECTIVE PLATINUM (II) COMPLEXES

M. Carrara, L. Cima, S. Zampiron, M. Nicolini, L. Sindellari and L. Trincia

INTRODUCTION

During the latest years a remarkable interest has grown about the use of sodium diethyldithiocarbamate (NaDEdtc) against cisplatin (DDP) toxicity (1,2,3). We observed a decrease of cytotoxicity of DDP when combined with NaDEdtc in a series of "in vitro" experiments on B16-F10 cells (4).

It is well known that diethyldithiocarbamate S-methylester (DEDTM) is the first metabolite of NaDEdtc formed in liver and in kidney (5,6). While NaDEdtc reacts with Pt(II) as a chelating bidentate species giving $Pt(DEdtc)_2$, DEDTM acts either as bidentate through both the sulfur atoms, giving the 1:1 complex $Pt(DEDTM)Cl_2$, or as unidentate through the thiocarbonyl sulfur, giving the complex [PtCl(DEdtc)(DEDTM)], with the ligand partially demethylated (7). While the search for new detoxicant substances continues, there is a great deal of effort aimed to elucidate the action mechanism of dithiocarbamates, also in relation to the biological versatility of these compounds.

Here we report the results of a cytotoxicity study on B16-F10 cells exposed to DEDTM and $Pt(DEDTM)Cl_2$ in which their activity was compared with NaDEdtc and its adduct $Pt(DEdtc)_2$.

MATERIALS AND METHODS

Preparation of the compounds.

Reagents were $PtCl_2$ (Johnson Matthey) and NaDEdtc (C.Erba). DEDTM was prepared, as previously reported (8), by reacting NaDEdtc and MeI in $EtOH/H_2O$. $Pt(DEdtc)_2$ and $Pt(DEDTM)Cl_2$ were prepared by reaction of $PtCl_2$ with NaDEdtc and DEDTM in acetone, respectively (7).

In vitro cytotoxic activity.

The experiments were performed using F10 metastatic cells of B16 murine melanoma (Fidler's source). The cells were seeded at $3 \cdot 10^4$ cells/ml in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1% 200 mM glutamine, 1% Hepes buffer, 100 U/ml penicillin, 500 μg/ml kanamycin. Each experiment was set up in triplicate in Petri dishes. The cultures were exposed to the four dithiocarbamates, incubated in an atmosphere containing 5% CO_2 at 37°C, recovered from culture dishes by 0.25% trypsin, neutralized by foetal calf serum, centrifuged at 1000 rpm for 10 min and counted in a haemocytometer.

DEDTM, $Pt(DEdtc)_2$ and $Pt(DEDTM)Cl_2$ were dissolved in a mixture of Cremophor EL : 94% ethanol, 1:0.42, diluted 1:100 in saline and finally 1:10 in the culture medium (8). DEdtc as sodium salt was dissolved in saline and diluted in medium.

The cells growth were tested at 10^{-4}, 10^{-5} and 10^{-6}M after 24 and 48 hrs exposure.

Cells survival was evaluated in the culture exposed to cisplatin (DDP) 10^{-5}M for 1 hr, washed three times, incubated for 1.5 hrs with growth medium and then exposed to medium containing NaDEdtc or DEDTM (10^{-3}M) for 1 hr (9).

All the results have been reported as percent of controls and statistically evaluated by Student's t test.

Optical microscopy.

Controls and cells treated with NaDEdtc or DEDTM or $Pt(DEdtc)_2$ or $Pt(DEDTM)Cl_2$ 10^{-4}M were cultured in duplicate on coverslips in Petri dishes and fixed in Bouin's fluid after 24 and 48 hrs, stained with haematoxylin-eosin, serially dehydrated in alcohol and cleared in xilene, mounted in Canada balsam and observed at optical microscopy.

Scanning electron microscopy (SEM).

After 48 hrs incubation at 37°C with the four compounds at 10^{-4}M, the cells were washed in normal medium and fixed for 30 min at 37°C, 2 hrs at room temperature and 2 days at 4°C in 2.5% glutaraldehyde in 0.1 sodium cacodilate buffer with 0.1 M sucrose (10). The fixed cells were then washed in medium for 30 min at room temperature. Finally, the cells have been postfixed in 1% OsO_4 in pH 7.2 buffer for 1 hr, washed several times in distillated water, dehydrated through a graded ethanol series, dried by CO_2 critical point procedure in Balzers Union, coated with gold using Edwards S150A Sputter Coater and examined at SEM (Cambridge Stereoscan 250).

RESULTS AND DISCUSSION

In vitro cytotoxic activity.

A statistically significant inhibition of cell growth was observed after 24 and 48 hrs exposure to the four compounds at 10^{-4}M. Only NaDEdtc and DEDTM showed a significant inhibition at 10^{-5}M after both 24 and 48 hrs exposure. No statistically significant inhibition was observed at 10^{-6}M (Fig. 1).

The exposure to cisplatin (DDP) alone caused a slight inhibition of cell growth. When the same cultures were afterwards treated with the two dithiocarbamates, only with DDP plus DEDTM a cytotoxic effect was still observed (Fig. 2).

Optical microscopy.

The cells exposed for 24 hrs to NaDEdtc were seriously damaged : nuclear lesions as pyknosis, shapeless cells, small size ones were observed. The cells exposed for 48 hrs to NaDEdtc were not examined because too seriously damaged.

The cultures treated for 24 and 48 hrs with DEDTM, $Pt(DEdtc)_2$ and $Pt(DEDTM)Cl_2$ showed no detectable modifications.

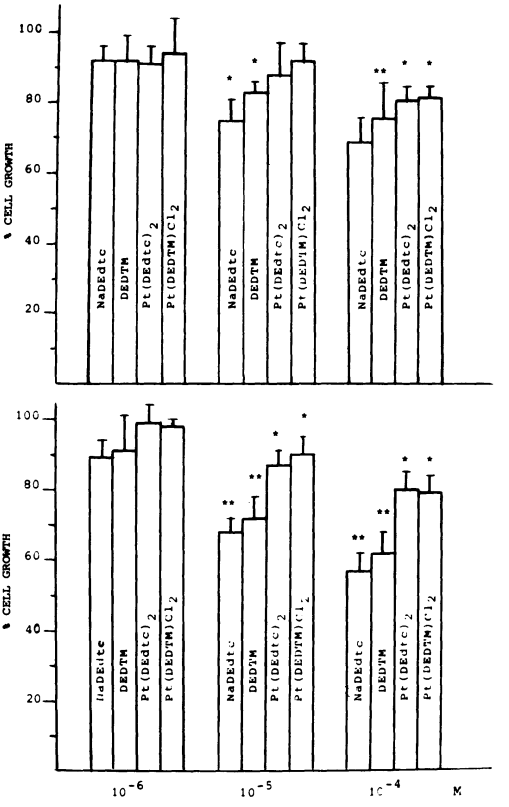


FIG. 1. Effects on cell growth after 24 hrs (upper panel) and 48 hrs (lower panel) treatment with the four dithiocarbamates. (*$p<0.05$; **$p<0.01$).

Scanning electron microscopy (SEM).

The cells exposed to NaDEdtc were not examined because too seriously damaged.

Changes in surface morphology were not observed in cells treated with DEDTM, while the cells exposed to both Pt-adducts appeared very extended and flattened (Fig. 3).

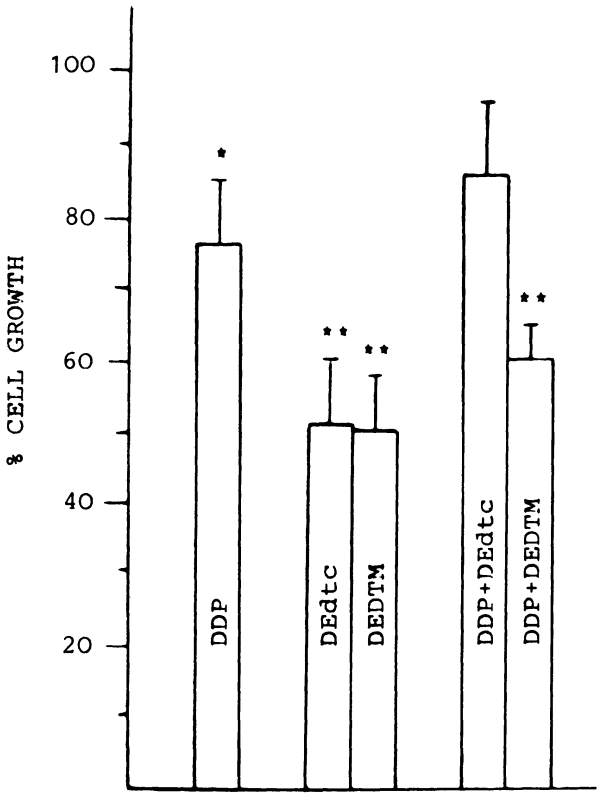
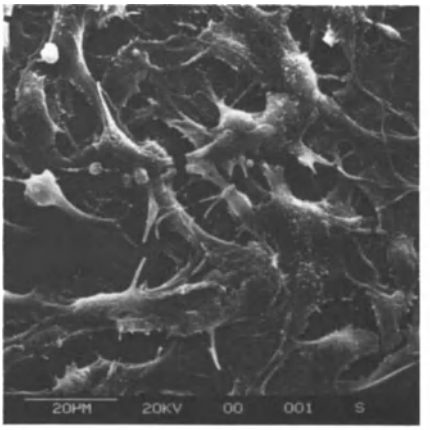
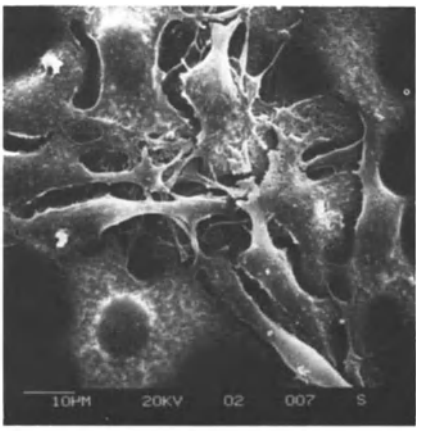


FIG. 2. Effects on cell growth of 10^{-5}M of DDP for 1 hr, washed three times, incubated for 1.5 hr with medium, then exposed to 10^{-3}M of DEdtc or DEDTM. (*$p<0.05$; **$p<0.01$).



A



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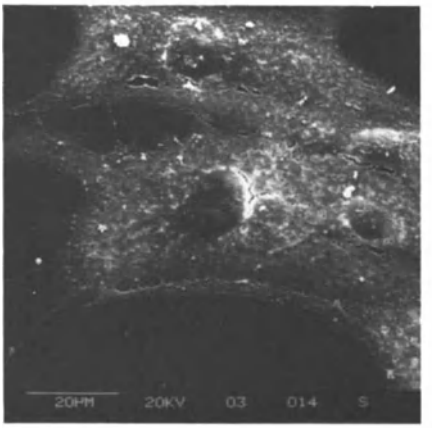

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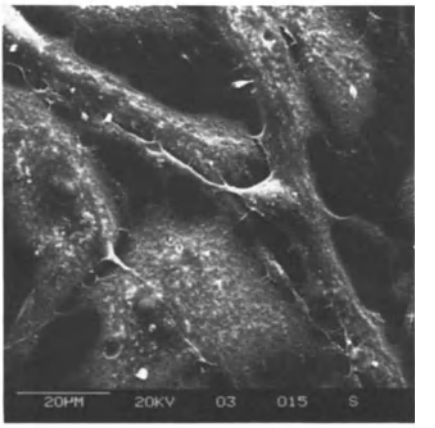

D

FIG. 3. Scanning electron micrographs of F10 cells cultures for 48 hrs. A = control cells; C = cells exposed to DEDTM; C = cells exposed to $Pt(DEdtc)_2$; D = cells exposed to $Pt(DEDTM)Cl_2$.

In conclusion, even though DEDTM is the first metabolite of NaDEdtc, it shows a cytotoxic activity similar to the parent compound. Therefore its higher lipophilicity does not seem to be essential for the cytotoxic response. On the other hand, a S-demethylation reaction cannot be excluded consistently with the hypothesized DEdtc-DEDTM interconversion "in vivo" (5).

It is noteworthy that other dithiocarbamates, such as sodium N-methyl-N-carboxy-methyl dithiocarbamate (Na_2MAdtc), which forms hydrosoluble adducts with Pt(II), caused a cell growth similar to NaDEdtc (11). Na_2MAdtc may be an alternative drug for obtaining "in vivo" either an antitumor synergistic effect or a toxic antagonism in cisplatin-chelating agent combined treatments. The polar character of the compound would lower the platinum redistribution in lipid rich tissues (11).

To avoid metal redistribution, another dithiocarbamate, the dihydroxylethyldithiocarbamate (NaDHdtc), more polar than NaDEdtc, has been proposed for mobilizing renal metal depots (12). Both Na_2MAdtc and NaDHdtc either inhibit the growth of tumor cells or counteract cisplatin cytotoxicity in a similar way but show different cytotoxic effects (11).

Since the more lipophilic adducts, $Pt(DEdtc)_2$ and $Pt(DEDTM)Cl_2$, still maintain a cytotoxic activity, it will be interesting to investigate the "in vitro" activity of Pt adducts with Na_2MAdtc and NaDHdtc because of their hydrophilic character. The S-methylesters, MADTM and DHDTM, probable metabolites of the anions, and their Pt(II) adducts will be studied.

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ENZYMURIA IN CISPLATIN-INDUCED KIDNEY DAMAGE AS AN INDEX OF ITS REVERSAL BY COMBINED TREATMENT WITH DIETHYLDITHIOCARBAMATE

M. Carrara, S. Zampiron, G. Bressa, L. Cima, F. Carmignoto, P. Gori, M. Zaninotto, C.D. Paleari and A. Burlina

INTRODUCTION

The nephrotoxicity of cis-dichlorodiammine platinum (II) (DDP) is the most serious dose-limiting factor for its therapeutic use. The important finding by Borch and Pleasants (1) that sodium diethyldithiocarbamate (DDTC) could partially inhibit DDP nephrotoxicity without adverse effects on tumor response has been confirmed by us (2,3) and by several other investigators (4,5). Since the nephrotoxicity of DDP is related to the damage of proximal tubule, our interest was focused on the earliest signes of this damage. Enzyme excretion has been proposed to be a sensitive noninvasive, nondestructive test of renal toxicity (6-8). Urine enzymes studies have chiefly focused on enzymes of lysosomal or intracellular origin. These enzymes are widely distributed and, because of their intracellular localization, their urinary excretion might be expected to increased only with cell death and lysis. Therefore we chose N-acetil- β-glucaminidase (NAG), a lysosomal enzyme (9) and α-glucosidase (α-GLUC), an enzyme contained only in the lysosomes of proximal renal tubular cells (10), as markers of DDP and DDTC toxicity after single administration and three combined treatments in

which DDTC was administered 1 hr before or 1 and 5 hrs after DDP, respectively.

MATERIALS AND METHODS

Animals and treatments.

Male Fischer 344 rats (Charles-River) weighing 125-150 g were used. The animals were divided into six groups and were kept in single metabolic cages to allow separate collection of urine and feces. The first group was used as control and enzyme levels in this group were taken as 100%. The other groups were treated with a single injection of DDP (10 mg/Kg s.c.) (Bristol, USA) or of DDTC (500 mg/Kg i.p.) (C. Erba, Italy), or with various combinations in which DDTC was administered 1 hr before or 1 and 5 after DDP, respectively. The animals were observed for a week and the urine collection was performed at 12 hrs intervals to evaluate enzymuria and creatininuria.

Urine enzymes analysis.

Urine samples (12 and 24 hrs) were collected over ice. The activity of NAG was determined by the method of Gressner and Roebruck (11), while the activity of α-GLUC was determined by the method of Ceriotti and Guarnieri (12). Urinary enzyme activity were calculated as units per liter and the changes after treatments were expressed as percentage of control. Creatinine was measured colorimetrically by Jaffe reaction using the method of Heinegard and Tiderström (13) and the changes after treatments were expressed as percentages of control.

Statistical analysis.

Statistical differences were calculated using Student's t test.

RESULTS AND DISCUSSION

Fig. 1 shows the variations of urinary NAG and α-GLUC output

during the five treatments. The delayed increase of enzymuria was statistically significant for DDP (Fig. 1,A), confirming the generic vulnerability of all the kidney tubules (NAG) (14) and the selective lesions of lysosomes in the proximal tubular cells (P_3 segment?) (α-GLUC) (15).

Urinary enzymes rose more slowly and a to a lesser extent after DDTC treatment, but, like after DDP treatment, NAG changes were higher in comparison with α-GLUC (Fig. 1, A and B). Therefore an increased enzyme excretion in terms of tubular cell pathogenesis was generally induced by either DDP or DDTC, but α-GLUC changes showed that the proximal tubule is less vulnerable to DDTC that to DDP. Thus the low nephrotoxicity of DDTC might be regarded as part of the typical kidney damage induced by other sulphur nucleophilic chelating agents, such as cysteine and N-acetyl--cysteine (which are unable to prevent DDP nephrotoxicity) and thiourea and thiosulphate (which interfere with the tumouricidal activity of DDP) (16).

After combined treatments no significant change was observed following DDTC pretreatment (Fig. 1, C), even if the time was analogous to that observed after DDP or DDTC alone. Therefore a mutual decrease of the early tubular damage of both drugs seems to occur, so that this phase of DDP nephrotoxicity is almost prevented. Unfortunately this DDTC rescue seems to be associated with a reduced DDP's antitumor response (17). On the other hand, the chemoprotection afforded by DDTC administered before DDP appears to be similar to that caused by thiosulphate, namely it is not related to the removal of platinum complexed with biological ligands, but rather results from a direct interaction of the molecule with free platinum species before they reach sites of toxicity (16).

On the contrary, DDTC post-treatments (Fig. 1, D and E) always caused an early but transient (within 12 hrs) increase of

enzymuria, which was higher when the interval between the two administrations was larger (5 hrs) (Fig. 1, E). These results account for a fleeting tubular damage in agreement with the findings of lower peak of blood urea nitrogen (BUN) in DDTC post-treated rats (18) and mice (5). Since BUN is not always an accurate index of glomerular filtration rate (GFR), enzymuria, the primary involvement of proximal tubule, was compared with the urinary creatinine (UCR), as a better index of GFR (Fig. 2).

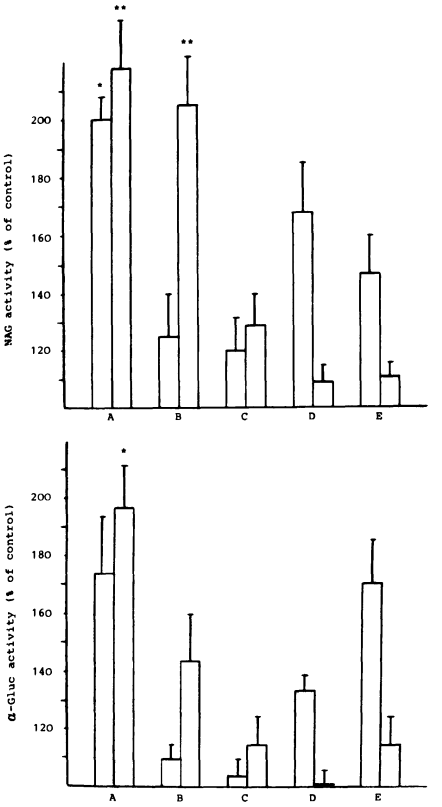

Fig. 1. Changes in urinary N-acetyl-β-glucaminidase (NAG) and α-glucosidase (α-Gluc) after 12 and 24 hrs of treatments. A = DDP 10 mg/Kg s.c.; B = DDTC 500 mg/Kg i.p.; C = DDTC 1 hr before DDP; D = DDTC 1 hr after DDP; E = DDTC 5 hrs after DDP.

Unexpectedly, while DDP alone caused an acute polyuric renal failure (15) with a significant increase of UCR (Fig. 2, A) and DDTC alone caused no UCR change (Fig. 2, B),a delayed decrease of UCR occurred following the combined treatments, that was significant after DDTC post-treatments (Fig. 2, D and E). Thus, in the last combined treatments, after 12 hrs an increased enzymuria is not associated with changes of UCR (early but transient acute tubular damage), while after 24 hrs a normalized enzymuria and an

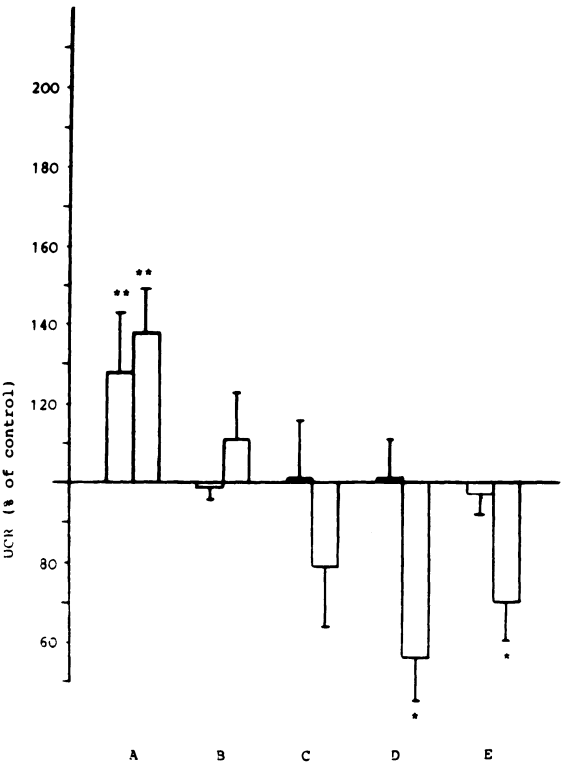

Fig. 2. Changes in urine creatinine (UCR) after 12 and 24 hrs treatment. A = DDP 10 mg/Kg s.c.; B = DDTC 500 mg/Kg i.p.; C = DDTC 1 hr before DDP; D = DDTC 1 hr after DDP; E = DDTC 5 hr after DDP.

UCR decrease coexist (delayed and more persistent general kidney damage). Since DDP produces renal failure in two distinct phases, i.e. an induction phase and a maintenance phase (15), it seems conceivable that the latter one after DDTC post-treatments might be not ascribed to interaction products of DDTC with the principal hydrolysis product of DDP (metabolite F or cis-diammine diaquo-platinum (II)) or with the mixed metabolites of DDP which are more nephrotoxic than DDP (19), but to platinum-DDTC chelates which are lipophilic. In fact, like Cd-DDTC (20) and Pb-DDTC (21) chelates, the Pt-DDTC adducts might be stored in some tissues, including kidney, but their instability might cause redistribution of platinum. In contrast to other heavy metals, the platinum atom may not mediate DDP toxicity and then the Pt-DDTC chelate might be paradoxically more nephrotoxic than platinum, like other lipophilic chelates of heavy metals (22).

In summary, the presented data indicate that DDTC administered before DDP prevents the early tubular damage but causes a possible reduction of antitumor response (17). By contrast no significant differences in this response have been recently reported after DDP treatment with or without DDTC administration 1 to 4 hrs after DDP (both i.p.) (5). It should be noticed that in our experiments these administration sequences still cause a moderate and transient tubular damage, even if DDP was administered s.c.

In conclusion, DDTC should be administered within 2 hrs from DDP treatment in order to maximally prevent nephrotoxicity without reducing the antitumor response to DDP to any significant extent.

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BIOTRANSFORMATIONS OF PLATINUM COMPOUNDS WITH THE 1,2-DIAMINOCYCLOHEXANE CARRIER LIGAND IN CULTURED L1210 LEUKEMIA CELLS

S.G. Chaney, S.K. Mauldin and G. Gibbons

INTRODUCTION

We have recently described a 2 column HPLC technique for separating and quantitating the biotransformation products of platinum compounds with the 1,2-diaminocyclohexane (dach) carrier ligand (1). We have also developed a combination HPLC-DNA binding assay which allows quantitation and identification of reactive platinum biotransformation products (Mauldin *et al.*, manuscript submitted for publication). We report here the application of these techniques to the study of two second generation platinum compounds with the dach carrier ligand. These studies have been carried out in tissue culture medium and with cultured L1210 cells.

1,2-Diaminocyclohexanemalonatoplatinum(II)[Pt(mal)(dach)] was chosen as a model compound for studying the mechanism of activation of platinum compounds with bidentate leaving ligands. Previous studies of platinum (II) compounds with bidentate leaving ligands have emphasized the contrast between the stability of the bidentate leaving ligand *in vitro* ($t_{1/2}$ > 11 days in water) and the apparent reactivity of these bidentate platinum compounds *in vivo* (2). However, none of these studies have actually measured the stability of these compounds in tissue culture medium (or in any other reaction mixture resembling *in vivo* conditions).

Similarly, 1,2-diaminocyclohexanetetrachloroplatinum(IV) [tetraplatin] was chosen as a model compound for studying the mechanism of activation of platinum (IV) anticancer agents. Platinum (IV) compounds are relatively unreactive with respect to

ligand exchange reactions (3). Thus, they are thought to be reduced to the platinum (II) level before reacting with DNA (4,5) although this hypothesis has recently been questioned (6). Pendyala *et al.* (7) have reported evidence for platinum (II) biotransformation products of cis-dichloro-*trans*-dihydroxy-bis-isopropylamineplatinum(IV) [CHIP] in the urine of dogs treated with that drug.

The 1,2-diaminocyclohexane ligand can exist in three different conformations which have slightly different therapeutic effectiveness in most cell lines (8). We have chosen to use the d,l-*trans*-1,2-diaminocyclohexane isomers because they are easier to prepare in radiolabeled form and they are usually more effective than the *cis* isomer in the L1210 cell line (8).

MATERIALS AND METHODS

Materials

Pt(mal)(dach) and tetraplatin were prepared with high specific activity, non-exchangeable tritium in the 4,5 positions of the 1,2-diaminocyclohexane moiety. All syntheses of the labeled and unlabeled platinum compounds as well as the starting material, (d,l)*trans*-1,2-diaminocyclohexane, will be described elsewhere (Wyrick and Chaney, manuscript submitted for publication). The L1210 cell line was obtained from Dr. Alan Eastman (Eppley Institute for Cancer Research, Omaha, NE). Growth medium consisted of RPMI-1640 medium supplemented with 15% fetal bovine serum and penicillin/streptomycin. The cells were subcultured twice a week and were grown in a humidified incubator with an atmosphere of 95% air-5% CO_2.

Methods

The two column HPLC system used to separate the intracellular biotransformation products has been described in detail in a previous paper (1). The elution profiles were analyzed and plotted with the Spectrodata software package (Spectrofuge Corp., Carrboro, NC). The individual biotransformation products were identified by comparing their retention times on reverse phase and cation exchange HPLC with the retention times of *in vitro*

prepared standards. Prior to HPLC analysis, samples were filtered through an Amicon YMT membrane filter with a 30,000 molecular weight cut-off (Amicon Corp., Danners, MA) to remove protein-bound platinum.

For analysis of intracellular platinum biotransformation products, L1210 cells at 10^6 cells/ml were incubated with ^{3}H-Pt-(mal)(dach) at 37°C. After incubation, extracellular platinum drug was removed by centrifuging the cells out of the medium. The cells were washed twice with 13 ml of cold phosphate buffered saline. The final cell pellets were stored at -80°C until needed for HPLC analysis. The ultrafilterable fractions for HPLC analysis were prepared by resuspending the cell pellets in one milliliter of cold HPLC grade water and sonicating the cells on ice.

Quantitation of the reactive platinum species in the ultrafilterable fractions was determined directly after filtration by a modification of the DNA-binding assay of Johnson *et al.* (9). Samples (400 µl) were incubated at 25°C for various times with salmon sperm DNA at a final concentration of 200 µg/ml in 5 mM $NaClO_4$, pH 5.5. After incubation, the samples were cooled on ice and 600 µl of cold 10% TCA was added along with 100 µl of calf thymus DNA (1 mg/ml) as carrier. The samples were kept on ice for at least 15 min before centrifugation for 20 min at 1200 xg, 4°C. The supernatant was discarded and the DNA pellet was dissolved in 200 µl of 0.1 M NaOH. The DNA was reprecipitated with 200 µl of cold 10% TCA, followed by addition of 2 ml of cold 5% TCA. The precipitated DNA was again pelleted by centrifugation as above and the supernatant discarded. The final DNA precipitate was dissolved in 0.5 ml NCS Tissue Solubilizer (Amersham, Arlington Heights, IL). Solubilization of the DNA took approximately 1-2 hr at 37°C or overnight at room temperature. After complete solubilization, 5 ml of Neutralizer Cocktail (Research Products Internation, Elk Grove Village, IL) was added and the samples were counted for ^{3}H incorporation. Measurement of background incorporation into DNA was performed as above except that the samples were precipitated with 10% TCA immediately after adding the salmon sperm DNA.

RESULTS AND DISCUSSION

Chemical Transformations of Pt(mal)(dach) in Tissue Culture Medium. The $t_{\frac{1}{2}}$ for displacement of the malonate ligand from Pt(mal)(dach) in RPMI-1640 tissue culture medium was 9.5 hr at 37° (Fig. 1).

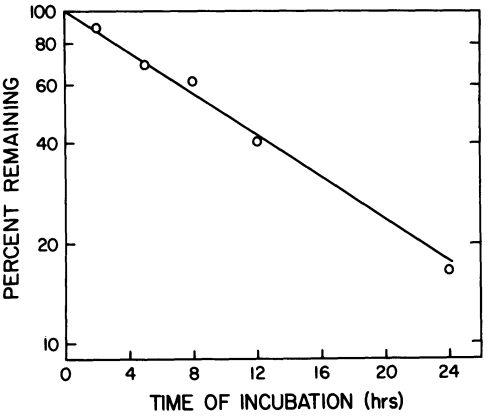

Figure 1. Stability of Pt(mal)(dach) in RPMI-1640 medium. 20 μg/ml [^{3}H-*trans*-dach]Pt(mal)(dach), 141 mCi/mmole, was incubated at 37° with RPMI-1640 medium + 15% Fetal Calf Serum in an atmosphere of 5% CO_2. At the times indicated, an aliquot was removed and filtered through an Amicon YMT membrane filter (30,000 MW cut-off). Pt(mal)(dach) was determined by reverse phase HPLC as described previously (1).

Of the inorganic anions present in the medium, chloride accounted for the greatest displacement of the malonate ligand. However, at the concentrations with which it is found in tissue culture media and in blood, bicarbonate was nearly as effective as chloride at displacing the malonate ligand. This observation is of particular significance because the bicarbonato-platinum complex is unstable and results in the formation of the biologically active aquated platinum complexes. At the concentrations with which they occur inside the cell, phosphates may play a similar role. Of the amino acids present in the media, the sulfur-containing amino acids were 50-400 fold more effective at displacing the malonate ligand than the other amino acids in RPMI-1640 medium. The HPLC separation of the transformation products which accumulated when

Pt(mal)(dach) was incubated in RPMI-1640 medium is shown in Fig. 2. The identification of these transformation products was carried out by comparing their retention times under three different HPLC conditions with the elution times of a series of 18 standards prepared *in vitro*.

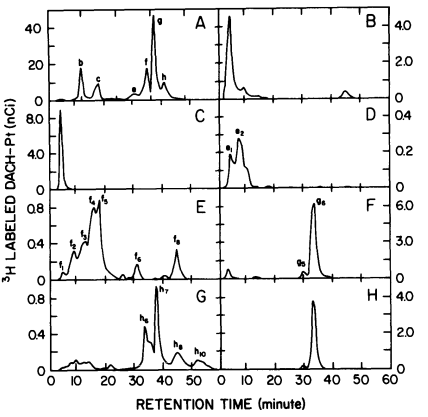

Figure 2. Major Transformation Products Formed from Pt(mal)(dach) in RPMI-1640 Medium. 20 μg/ml [^{3}H-*trans*-dach]Pt(mal)(dach), 141 mCi/mmole, was incubated at 37° for 24 hr with RPMI-1640 medium and an aliquot of the reaction mixture analyzed by the two column HPLC separation system described previously (1). The nomenclature used in labeling the peaks is based in part on the more complex elution profiles observed in studies of intracellular biotransformation products. Fig. 2A, reverse phase elution profile; Fig. 2B, cation exchange elution profile of peak b; Fig. 2C, cation exchange elution profile of peak c; Fig. 2D, cation exchange elution profile of peak e; Fig. 2E, cation exchange elution profile of peak f; Fig. 2F, cation exchange elution profile of peak g; Fig. 2G, cation exchange elution profile peak h; Fig. 2H, cation exchange elution profile of the 1,2-diaminocyclohexanemethionine-platinum (II) standard. All cation exchange separations shown in this Figure were at pH 4.

The major transformation products formed were 1,2-diaminocyclohexanemethioninepLatinum(II) (38%), other amino acid-platinum complexes (19%), 1,2-diaminocyclohexanedichloroplatinum(II)[$PtCl_2$-(dach)] (14%), and aquated platinum complexes (6.4%). Eleven percent of the Pt(mal)(dach) remained intact. The 1,2-diaminocyclohexanemethioninepLatinum(II) complex appeared to be essen-

tially inactive, as judged by an extremely low rate of uptake and little or no binding to the cell membrane of L1210 cells.

Intracellular Biotransformations of Pt(mal)(dach) in Cultured L1210 Cells. In L1210 cells cultured in RPMI-1640 medium plus 15% fetal calf serum the $t_{\frac{1}{2}}$ for Pt(mal)(dach) was 28 min at 37°. This rate of breakdown is consistent with the displacement rates of the malonate ligand by various amino acids (which we have determined *in vitro*) and previous estimates of the intracellular amino acid concentrations. Thus, there would appear to be no necessity for invoking enzymatic activation of platinum compounds with bidentate leaving ligands. A combination of reverse phase HPLC and a DNA binding assay were used to identify and quantitate the reactive biotransformation products. 1,2-Diaminocyclohexane-aquachloroplatinum(II)$[Pt(H_2O)(Cl)(dach)]^+$ was the predominant reactive species at early times. However, at latter times (between 8 and 24 hr), one or more additional reactive biotransformation products accumulated in the cell.

Model for Activation of Pt(mal)(dach). The model we propose for the activation of Pt(mal)(dach) is shown in Fig. 3.

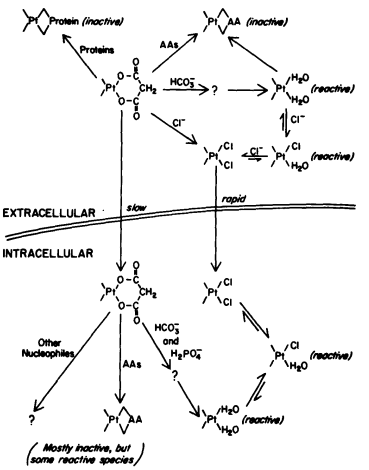

Figure 3.
Model for Activation of Pt(mal)(dach)

In RPMI-1640 medium we found that the malonate ligand was displaced at approximately equivalent rates by protein, amino acids (predominantly methionine), bicarbonate, and chloride at the

concentrations present in the medium. The platinum-protein and platinum-amino acid complexes were essentially inactive and were probably not taken up by the cell, based on our studies with the platinum-methionine complex. However, both the chloride and bicarbonate displacement reactions can be considered extracellular activation pathways. The chloride displacement reaction can be considered an activation pathway because $PtCl_2$(dach) is taken up 8 times more rapidly by L1210 cells than Pt(mal)(dach) (10); and, once inside the cell, $PtCl_2$(dach) should dissociate to form reactive aquated species (11). In fact, it appears likely that most of the intracellular $PtCl_2$(dach) arises from the extracellular displacement reaction. This conclusion is based on two observations. First, displacement of the malonate ligand by chloride is exceedingly slow at intracellular chloride concentrations. Secondly, the time course for $PtCl_2$(dach) accumulation and disappearance inside the cell closely parallels that seen in RPMI-1640 medium. The extracellular bicarbonate displacement reaction can also be considered an activation pathway because the bicarbonato complex is unstable and readily decomposes to give various aquated species. At the chloride concentrations which prevail in the medium, at least some of these aquated complexes would be converted to $PtCl_2$(dach) for entry into the cell.

Once Pt(mal)(dach) enters the cell, the malonate ligand is very rapidly displaced. The major portion of the observed intracellular rate of malonate displacement can probably be accounted for by the reaction of Pt(mal)(dach) with glutathione and amino acids without the necessity of invoking enzymatic activation. Most of the platinum-amino acid complexes which form in this manner appear to be inert. However, at intracellular concentrations, both bicarbonate and phosphates can effectively displace the malonate ligand as well, and both form unstable complexes which dissociate to give rise to aquated species. Thus, these reactions can be considered to be major intracellular activation pathways leading to the production of $[Pt(H_2O)(Cl)(dach)]^+$. The slow accumulation of reactive platinum species other than $[Pt(H_2O)(Cl)(dach)]^+$ is most likely due to the displacement of the malonate

ligand by some of the less reactive amino acids (and possibly by other less reactive nucleophiles as well). One might predict that relatively weak nucleophiles would react more slowly with Pt(mal)(dach) and, once they did react, would tend to form unstable platinum complexes. In agreement with this hypothesis, our data show that those platinum-amino acid complexes which form most slowly (e.g. lysine and arginine) do have significant reactivity towards DNA.

Chemical Transformations of Tetraplatin in Tissue Culture Medium. In analogy to other platinum(IV) compounds, activation of tetraplatin is thought to involve an initial reduction to 1,2-diaminocyclohexanedichloroplatinum(II) [$PtCl_2$(dach)]. However, the rate and location of this activation reaction is not known. In experiments with L1210 cells in culture, tetraplatin and $PtCl_2$(dach) had nearly identical cytotoxicity (ID_{50} = 0.80 and 0.75 μM, respectively). In addition, the rate at which tetraplatin and $PtCl_2$(dach) inhibited DNA synthesis and cell growth was very similar. These observations suggested that the reduction of tetraplatin to $PtCl_2$(dach) was very rapid, but did not indicate whether the reduction was an intracellular or extracellular event.

Therefore, tetraplatin was incubated in RPMI-1640 medium plus 15% fetal calf serum at 37°. Tetraplatin and $PtCl_2$(dach) were resolved using the HPLC technique described by Anderson *et al.* (12). The other chemical transformation products in the medium were separated by reverse phase HPLC as described previously (1). These data are summarized in Fig. 4. Tetraplatin was converted to $PtCl_2$(dach) very rapidly ($t_{\frac{1}{2}}$ = 15 min) and the $PtCl_2$(dach) was then very slowly ($t_{\frac{1}{2}}$ > 5 hr) converted to other transformation products in the medium. As seen in Fig. 5, the transformation products which formed in the medium appear to be very similar to those observed previously when the platinum (II) compound 1,2-diaminocyclohexanemalonatoplatinum (II) [Pt(mal)-(dach)] was incubated with RPMI-1640. That is, most of the transformation products appeared to be amino acid complexes at the

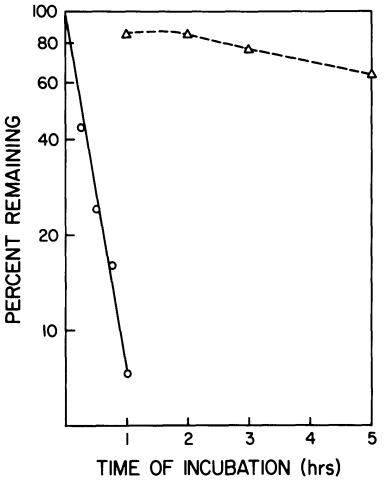

Figure 4: Time Course of Tetraplatin Transformations in RPMI-1640 Medium. 20 μg/ml Tetraplatin, 402 mCi/mmole, was incubated for the times indicated with RPMI-1640 medium. The quantitations of tetraplatin (0) and $PtCl_2$(dach) (Δ) were obtained from HPLC separations described previously (1,12).

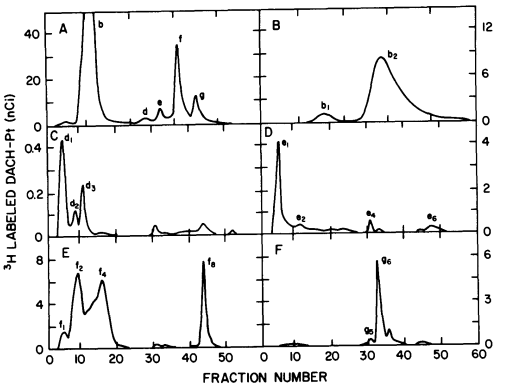

Figure 5: Major Transformation Products of Tetraplatin in RPMI-1640 Medium. ^{3}H labeled tetraplatin (419 mCi/mmole) at a concentration of 44 μM was incubated 2 hr at 37° in RPMI-1640 medium. Following filtration of the sample through an Amicon YMT membrane, aliquots of the sample were analyzed by the HPLC separation procedures described previously (1,12). Fig. 5A, reverse phase HPLC separation of incubation mixture on Whatman ODS-3 column (1) Fig. 5B, reverse phase separation of peak b on Zorbax colum (12); Fig. 5C, cation exchange separation of peak d (1); Fig. 5D, cation exchange separation of peak e; Fig 5E, cation exchange separation of peak f; Fig. 5F, cation exchange separation of peak g. Identifications: peak b_1 = tetraplatin, peak b_2 = $PtCl_2$(dach), peak e_1 = Pt(cystine)(dach), peak f_4 = Pt(serine)(dach), peak g_5 = free dach, and peak g_6 = Pt(methionine)(dach).

platinum (II) level. Thus, the data indicate that in RPMI-1640 medium, there is a rapid reduction of tetraplatin to $PtCl_2$(dach) followed by a much slower series of nucleophilic displacement reactions, similar to those seen previously for other platinum (II) compounds with the diaminocyclohexane carrier ligand. Since platinum (IV) compounds are much less reactive than platinum (II) compounds (3), this represents a pathway for the rapid activation of tetraplatin. RPMI-1640 medium contains no cysteine, $FeSO_4$ or ascorbic acid, and only 3 μM glutathione. Preliminary data indicate that the fetal calf serum is primarily responsible for the rapid reduction of tetraplatin, suggesting that the same rapid activation is likely to occur in the blood as well.

CONCLUSIONS

In summary, the techniques described in this paper allow the identification and quantitation of extra- and intracellular platinum biotransformation products, the determination of their reactivity towards DNA, and the half-life of the parent drug both in the culture medium and in the cell. These techniques make possible a number of potentially useful studies. For example, it is now be possible to study the detailed mechanism of activation of platinum drugs. It should also be possible to compare the biotransformation of platinum compounds in different cell types and identify differences in metabolism which might lead to the organ-specific toxicity of certain platinum drugs (13). The techniques described in this paper are suitable only for platinum compounds with the dach carrier ligand. However, we believe these techniques can be adapted to follow the intracellular biotransformations of any platinum compound which can be prepared with a radiolabeled carrier ligand (14) or with [195m]platinum (15). Application of these techniques to other platinum compounds will require the development of HPLC separation systems with suitable resolution (our data suggest two different columns will be required), preparation of a sufficiently large number of platinum standards, and development of a suitable measure of platinum reactivity, such as the DNA binding assay.

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THE RELATION BETWEEN PHAGE INACTIVATION BY AND DNA-BINDING OF Pten $(H_2O)_2^{2+}$ AND ITS CORRESPONDING BASES AT DIFFERENT ACIDITIES

E. Clausen, J. Josephsen, G. Kerszman and H.B. Pedersen

SUMMARY

Kinetics of the binding of $Ptenaq_2$ to DNA in vitro at pH 5.6, 6.2 and 6.65 is closely correlated to the kinetics of inactivation of T4Do and T7 phages under identical conditions.

The inactivation of a phage particle corresponds on average to binding to its DNA of 18(for T4Do) or 7(for T7) platinum complex molecules.

$Pten(H_2O)_2^{2+}$ binds to DNA and inactivates phages at a rate about 15 times higher than $Pten(H_2O)OH^+$.

INTRODUCTION

Studies of inactivation of bacteriophages by Pt(II) complexes provide one way to bridge a gap between investigations of the action of these compounds on the cellular level and the studies of their reactions with DNA in vitro.

While working on the cellular level it is impossible to control the reaction conditions such as pH, concentration of ions etc. One is also confronted with all the complexities of cellular metabolism, membrane permeabilities etc.

*en=1,2-ethanediamine. The equilibrium mixture of the three forms, $Pten(H_2O)_2^{2+}$, $Pten(H_2O)OH^+$ and $Pten(OH)_2$ will in the following be referred to as "$Ptenaq_2$" without charge or as "the diaqua complex" collectively.

On the other hand the investigator working with in vitro reactions between DNA and Pt(II) compounds is unable to observe directly the biological effects of these reactions.

A phage as a model system combines some advantages of in vivo and in vitro conditions.

We have reported earlier(1,2) the inactivation kinetics of T4 phage and the influence of pH, some buffers and ionic media upon this kinetics. The aim of the present study was to relate the rate of inactivation of phages T4Do and T7 at different acidities to the in vitro binding of $Ptenaq_2$ to DNA under identical conditions: pH, buffer and ionic medium.

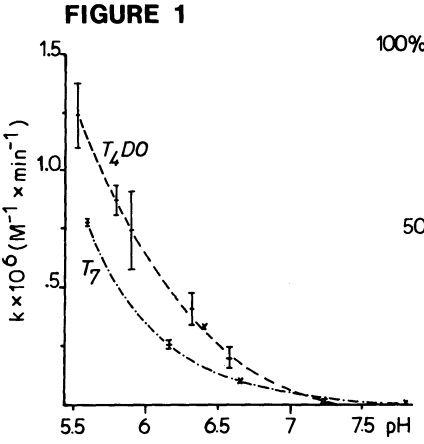
MATERIALS AND METHODS

Phage experiments: The osmotic shock resistant mutant T4Do(3) was kindly provided by The Institute of Microbiology, University of Copenhagen.

Chemicals, media, phage techniques and inactivation procedures, using Bis-TRIS or Hepes buffers (0.04 M. and 1.0 M. in $NaNO_3$) were the same as described earlier (2). DNA-binding experiments: Stock-solutions of ^{14}C- Pten $(H_2O)_2(NO_3)$ were synthesised essentialy as reported earlier for the non-labelled complex (2) using microscale techniques, details of which will be reported elsewhere. Calf thymus DNA was purchased from Sigma and used instead of phage DNA. The DNA binding and phage inactivation media were identical with respect to composition and pH. The binding experiments were performed at 37°C, essentially according to the procedure described Johnson et al (4) applying glass-fiber filters to collect the precipitated DNA. Radioactivity was measured using Dimilume-30 (Packard) scintillation liquid and a Nuclear Chicago Mark II counter.

RESULTS

Fig. 1 depicts the relationship between the second order rate constants k_i for inactivation of T4Do and T7 bacteriophages and pH.



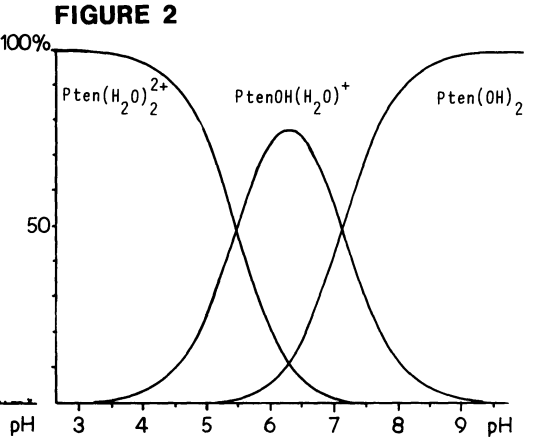


Figure 1. Second order inactivation rate constants as a function of pH of T4Do and T7.

The Pseudo-first order inactivation rate constants has been calculated on the basis of the exponential part of the survival curves (not shown) by linear regression according to Hald (5) for $v^2<1$. The dispersion of the calculated values based on at least three independent experimental sets of data is shown.

Figure 2. Relative concentrations of $Pten(H_2O)_2^{2+}$, $Pten(H_2O)(OH)^+$ and Pten(OH) as a function of pH. Medium: 1M $NaNO_3$.

It has been shown previosly that a linear relationship exists between the pseudo-first order inactivation constants and the concentration of $Ptenaq_2$. The calculation of overall second order rate constants is thus justified. It can be seen from Fig. 1 that for both bacteriophages the k_i decreases when pH increases.

In order to find out if this dependance relates to

the kinetics of $Ptenaq_2$ binding by DNA, binding of ^{14}C labelled $Ptenaq_2$ to calf thymus DNA was measured at pH 5.60, 6.20 and 6.65 under the same conditions (buffer, temperature and ionic medium) as for the bacteriophage inactivation experiments.

Nucleotide/$Ptenaq_2$ ratios R_i ranged from 0.2 to 0.005 and in all cases the binding decreased with increasing pH. Assuming the reaction to be pseudo-first order in nucleotide, the second order binding constant were calculated.

Table 1. K_b and k_i values for given pH value.

	k_b DNA	k_i T7	k_i T4 Do
pH 5.60	64	7.8×10^5	1.2×10^6
pH 6.20	29	2.6×10^5	0.47×10^6
pH 6.65	12	1.0×10^5	0.18×10^6

Inactivation constants k_i for T7 and T4Do reaction with $Ptenaq_2$ as well as the binding constants for the reaction between calf thymus DNA and the diaqua complex, k_b values are given for experiments using [DNA-nucleotides] = 10^{-3}M and [$Ptenaq_2$] = 5×10^{-6}M. K_i and k_b are in $min^{-1}M^{-1}$.

These are given in table 1, together with the corresponding second order inactivation constants. The relative concentrations of $Pten(H_2O)_2^{2+}$, $Pten(H_2O)OH^+$ and $Pten(OH)_2$ as a function of pH are depicted in Fig. 2.

Comparisons of the data of Fig. 1 and Fig. 2 together with table 1 clearly demonstrates that decreasing inactivation and binding constants are related to decreasing concentration of $Pten(H_2O)_2^{2+}$.

DISCUSSION

The results presented in Fig. 1 and Tab. 1 show a close correlation between the inactivation rate constant changes for T4Do and T7 and binding rate constant changes for DNA, as a function of pH.

For both phages the values of correlation coefficient $r>0.995$. The regression analysis of the dependance of the inactivation rate constants k_i(T4Do) and k_i(T7) on the binding constant k_b at different values of pH gives: k_i(T4Do)$=1.8 \times 10^4 \times k_b$ and k_i(T7)$=1.1 \times 10^4 \times k_b$. A simple model would predict the relationship: $k_i/k_b=[N_y]/[N_i]$ where N_y=nucleotide content of a phage particle and N_i= the average number of nucleotides bound to a Pt complex per inactivation event. This gives N_i(T4Do)=18 and N_i(T7)=7. The last value agrees reasonably well with the value of 4 obtained by Shooter et al (6) using a different method and quite different experimental conditions.

As inactivation experiments do not point to a multi-hit inactivation kinetics, the straigtforward interpretation of N_i, is that it represents the average ratio of nonlethal to lethal adducts.

Assuming simply that the lethal adducts are intra-strand cross-links between adjacent guanine bases, from the difference in GC content in DNA of the phages considered one would expect N_i(T4Do) to be only ca 4/3 of N_i(T7). The actual difference defies this oversimplified explanation.

An obvious alternative interpretation relies on the possible differences of repair of Pt adducts after infection of cells by T4Do and T7.

This explanation is in apparent conflict with our previous results showing that Pt(II) complexes impair the penetration of T4 DNA into the infected cells. However this result were obtained with osmotic shock sensitive T4 wild type which is much more resistant to the lethal action of Pt(II) complexes. At present it can not be ruled out that inactivation of the wild type, which prevents DNA injection, depends on reactions involving proteins and that Pt-inhibited T4Do as well as T7 can inject their DNA after all. This matter needs obviously further investigation.

Second order rate constants for the reaction between DNA and $Ptenaq_2$ reported here are much lower than those obtained by others (4,6). This can be explained by the differences in reaction conditions. We used high saltconcentration and bis-tris buffer in order to define pH of the reaction medium as accurately as possible.

The multiple regression analysis of the rate constants as a function of the ratio of $Pten(H_2O)_2^{2+}$/$Pten(OH)(H_2O)^{+}$ at different values of pH (Fig. 2) shows that contribution of the two ions to the observed reaction rate is about 15:1 respectively. This conclusion relies on two assumptions:

1. That $Pten(OH)_2$ does not react with DNA. This assumption seems to be safe enough and in agreement with the conclusions of other authors.
2. That pH influences the reaction only through its effect on ionization of the complex. This assumption is obviously more questionable. The possible effect of pH on the reactivity of DNA with platinum complexes needs further investigation.

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PROTECTIVE EFFECT OF PROCAINE AGAINST CISPLATIN INDUCED NEPHROTOXICITY IN MICE

M. Esposito, R.A. Fulco, A. Zicca, A. Cadoni, R. Rosso and A. Sobrero

Clinical data suggest a dose response curve for cisplatin (DDP) in a number of human neoplasm. In an attempt to increase the therapeutic index of this drug, a host of compounds that reduce DDP nephrotoxicity allowing the administration of higher doses of the drug is under study; concern however exists that reduction of toxicity may also reduce the antitumor activity of DDP. Procaine is a local anesthetic drug that modifies several membrane-mediated cellular processes and in experimental tumor systems has been shown to potentiate antineoplastic agents in vitro. In addition, based upon our previous observations that a) procaine protects V79 hamster cells from the mutagenic effects of DDP without decreasing the cytotoxicity of this drug in vitro and b) procaine does not alter the sensitivity of human colon carcinoma cells, HCT-8 to DDP in vitro, we have tested the lethality and efficacy of DDP, procaine and their combinations in vivo.

Procaine was non-toxic at doses ranging between 20 and 100 mg/Kg given IP on day 1 or 1 and 5 to normal BDF1 mice, and the LD_{50} value was 222 mg/Kg for the single IP administration. The simultaneous administration of DDP + procaine (40 mg/Kg) produced LD_{50} and LD_{90} values of 23 and 38 mg/Kg, respectively, that are approximately 2 times higher than the values observed with DDP alone.

The efficacy of IP DDP + procaine combinations on the survival of BDF1 mice injected with 10^5 P388 leukemic cells on day 0 was essentially identical at subtoxic doses of DDP ($\leq LD_{10}$) whereas 60 to 80% higher doses of DDP were administrable simultaneously with procaine without lethal toxicity. These intense regimens produced a significant increase in survival as could be expected from a dose-response curve. This is evident both in the single day, 2-day and 3-day treatments where a total of 33 over 122 (27%) mice were cured (60 day survivors) by combination DDP + procaine as compared to only 6/76 (8%) animals cured by DDP alone (Table 1).

Table 1. Procaine effect on toxic doses of DDP*.

Treatment	MST° (days)	Range	%ILS¨	%Wt loss at day 7	60-day survivors
Control (saline)	12.0±0.6	10-18	-	+ 4.9	0/46
Procaine, P (40 mg/kg)	11.8±0.3	10-17	-	+ 5.3	0/29
Day 1					
10 mg/Kg DDP	17.5	6-36	+ 46	-15.2	3/18
10 mg/Kg DDP+P	27.5	13-32	+129	- 9.0	7/18
15 mg/Kg DDP	5.0	3-5	- 58	-	0/8
15 mg/Kg DDP+P	36.0	26-36	+200	-18.7	3/8
25 mg/Kg DDP+P	9.5	6-33	- 21	-29.4	1/8
Days 1, 5					
8.0 mg/Kg DDP	8.5	6-45	- 30	-29.3	3/35
8.0 mg/Kg DDP+P	38.6	11-45	+217	-17.8	16/44
10 mg/Kg DDP+P	60+	11-45	>400	-21.1	5/10
12.5 mg/Kg DDP+P	9.0	8-25	- 31	-26.1	0/9
15 mg/Kg DDP+P	7.0	6-24	- 46	-21.5	1/10
Days 1, 5, 9					
8.0 mg/Kg DDP	8.5	7-11	- 27	-27.4	0/15
8.0 mg/Kg DDP+P	36.0	13-48	+200	-19.7	5/15

* 10^5 cells/mouse were injected IP on day 0.
° Median survival time, ± SD.
¨ % Increase in life span.

Preliminary data suggest a protective effect of procaine on DDP nephrotoxicity, as evaluated by serial BUN determinations and histopathological changes at the tubular level. The increased BUN values observed 4 to 7 days after a single DDP injection (either 8 or 16 mg/Kg) did not occur after the same doses of DDP + procaine.
Dose dependent degenerative changes from DDP were localized to the murine proximal tubules. The extent of tubular damage observed in mice treated with DDP 8 mg/Kg + P on days 1, 5 and 9 was comparable to the changes observed in mice that received only 1 dose of DDP alone (8 mg/Kg).
Finally procaine induced substantial changes in the plasma and tissue pharmacokinetics of total platinum as measured by flameless atomic absorption spectroscopy. Liver, kidney, spleen, stomach, intestine, heart and lung samples as well as plasma from mice treated with a single DDP dose of 8 mg/Kg contained approximately 1.5 times more total platinum than the samples from mice treated with DDP + P combination at the same dose.
These data indicate a possible protective effect of procaine on the nephrotoxicity of DDP and encourage further testing of DDP + P combinations in mice before its clinical use.

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ANTITUMOR ACTIVITY AND TOXICITY OF SELECTED PLATINUM COMPLEXES

F. Kiss, J. Vorlíček, J. Hejl, B. Hofírek, E. Hájek, R. Blechová, M. Bohuminská, J. Novotný and I. Závodná

An extensive preclinical testing of various types of platinum complexes, analogues of cisplatin, was started in Czechoslovakia in 1981. Compounds, containing chloride, sulphate, malonate and its derivatives, citrate, isocitrate, 4-carboxyphthalate and ascorbate as acidoligands, and NH_3, isopropylamine and cis-,trans-1,2-diaminecyclohexane (DACH) as aminoligands were synthesized.

Most of these compounds exhibited antitumour activity, comparable with that of cisplatin (ILS on P 388 leukemia were higher than 200%), which was demonstrated (1) in accordance with the previously published data. In the case of DACH, however, the expected higher antitumour activity of complexes containing trans-1,2-DACH was not confirmed. This is demonstrated for citrate complexes in Table 1.

Table 1. Antitumour effectiveness of diaminecyclohexane citrate platinum (II) complexes containing different fractions of cis-and trans-DACH stereoisomers on P 388 leukemia

Content of DACH isomers cis %	trans %	Optimal treat. dose $mg.kg^{-1}$	ILS %
0	100	2 x 20	244
30	70	2 x 20	255
70	30	2 x 20	239

As a result of screening carried out with a number of potential complexes three compounds were finally selected for further testing : oxoplatin (oxidized form of cisplatin), iproplatin (CHIP, NSC 256927), and carboplatin (CBDCA, NSC 241240). Extensive preclinical testing aimed at comparison of gastrointestinal, kidney and hematological toxicities was carried out on mice, rats and beagle dogs under various regiments of cytostatic administration with equitoxic doses corresponding to LD_0 values for each species of experimental animals.

It is only the hematological toxicity that can be considered higher as compared with cisplatin. Not only myelotoxicity (a decrease of thrombocyte and leukocyte counts), but also significant effects upon all development stages of erythropoiesis were observed.
Changes in the bone marrow preceded changes in the periphery blood (in proper time intervals). The regeneration ability of bone marrow was fully preserved in all species of examined animals, as demonstrated by a permanent presence of megakaryocytes and an unusually rapid return of thrombocyte values to the physiological limits. The same applied to the regeneration of leukocytes in the course of 1-2 weeks.
The highest but reversible changes in dogs were obserwed following the application of carboplatin (thrombocytopenia) and iproplatin (leukopenia).

During the study of chronic toxicity in dogs no significant changes in the values of creatinine and urine in blood were found. The alterations of kidneys upon the administration of all three complexes were of quite a different character than in the case of cisplatin toxic effect. The ultrastructural evaluation showed in individual cases greater or lower focal to diffuse glomerulonephritis without more pronounced cicatricial or membraneous changes. Electron-microscopical examination indicated that the cause of these renal alterations are

immunocomplexes deposits of which were found in the kidneys (2).

Gastrointestinal toxicity was found to be most pronounced in the case of iproplatin and it was manifested by the change of enzyme activity of the small intestine in rats, and by inappetence, thirst, and particularly by vomitus in dogs (increasing in the order carboplatin, oxoplatin, iproplatin).

Based on these results and on conclusions of other experimental studies, clinical testing of carboplatin was successfully under way since 1985 and phase I of a study of oxoplatin should begin shortly.

It can be assumed that tolerance to oxoplatin in man should not be worse than to cisplatin, iproplatin, and possibly carboplatin. If a therapeutic effect is found, then a high antimetastatic effect observed in experimental models would be desirable to exist also in humans; this remains, however, to be verified.

The clarification of both these questions will enable a more exact determination of the position which oxoplatin has among cisplatin analoques.

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THE INTERACTION AND DEGRADATION OF XANTHINE BASES WITH PLATINUM METALS

J.R. Lusty and H.S.O. Chan

In this study the interaction of nucleobases with some platinum metals has been investigated and their degradation has been studied using thermal methods. In particular the products of the interaction of cis-diamminedichloroplatinum(II) with the nucleobase xanthine and some of its methyl derivatives, including the model base 9-methylxanthine, have been characterised using spectroscopic methods. The degradation pathway, at elevated temperatures, for these complexes has been studied using Thermal Gravimetric Analysis (TGA), Differential Scanning Calorimetry(DSC) and Differential Thermogravimetry (DTG).

The degradation can be divided into two quite separate pathways, involving initial breakdown of the base followed by complex degradation. The first step is similar in both air and N_2 but in air the second reduction process is rapid and occurs at between 85-100% min^{-1}, leaving the metal oxide as a product, whereas in N_2 this second step is much slower resulting in a fully reduced product.

In complexes of the metals studied, Platinum(II), Palladium(II) and Rhodium(III), the initial step produces an imidazole complex as an intermediate, which rapidly degrades further. Activation energy studies show that the energy required for the initial breakdown is much higher than the subsequent degradation. Furthermore, it has been demonstrated that the energy required for this latter stage accurately reflects the

nature of the nucleobase-metal bond. The reaction is catalysed by two factors: (i) the metal centre, although this is not very specific and occurs for a range of transition metals, (ii) oxygen present in the system or access to an oxygen containing environment.

Strains induced within a complex, such as enforced bidentate behaviour of the base increase the rate of the degradation process. This clearly demonstrates a pathway by which base degradation and complex break-up can occur, and would be appropriate to a system where catalytic activity is present.

INTERFERENCE OF THIO-COMPOUNDS WITH *CIS*-DIAMMINODICHLOROPLATINUM (DDP) ON TUMOR CELLS *IN VITRO*

G. Magnolfi, S. D'Ancona and T. Berti

SUMMARY

Interference of thio-compounds with cis-DDP in vitro on metastatic subline of murine B16 melanoma was investigated.

Our results confirm a protective role exerted in vitro by Mesna on cis-DDP toxicity. CPDS and 2,2'-PDS, two "thione-forming" disulfides show, on the contrary, a synergic effect with cis-DDP: it suggests their possible role in sensitizing tumor cells to anticancer drugs (through a changing of cell surface, a reduction of GSH content and/or an inhibition of poly A formation). The possible mechanisms of the interactions between thio-compounds and cis-DDP are discussed.

INTRODUCTION

The protective effect exerted by sulfhydryl compounds such as diethyldithiocarbammate (1) and thiosulphate (2) on cis-DDP toxicity in vitro and in vivo is well known.

The role of Mesna, as a protective substance against cis-DDP, is not so clear : in vitro Mesna exerts a good protection on several cell lines (3,4,5), in vivo the results are not so univocal (3,6). The action mechanism of Mesna is mainly to scavange cis-DDP before it enters into the cells (7) as a chemical direct interaction.

CPDS and 2,2'-PDS are "thione-forming" disulfides. Their

molecules are not able to directly react with cis-DDP molecules and so the interaction is probably mediated by their action on different targets of the membrane or inside the cells.

2,2'-PDS freely enters into the cells and causes oxidation of several substrates as glucose-6-phosphate, glutathione and NADPH (8).

CPDS has two carboxylic groups, ionized at physiological pH. They prevent, to some extent, the CPDS entrance into che cells, so that CPDS mainly reacts with -SH groups of cell membranes, modifying them (9,10). On the other hand CPDS also oxidized cellular GSH (11). The cell surface modification induced by CPDS could explain its inhibitory action on cell adhesion in vitro (12) and its antimetastatic activity in vivo (13).

Furthermore 2,2'-PDS and CPDS, because of their structure, could be considered as nicotinamide analogs, i.e. inhibitors of poly (ADP-ribose) polymerase (13) inside the cells.

This paper describes the results of the interactions in vitro of several thio-compounds (2,2'-PDS, CPDS, 6-MNA and Mesna) with cis-DDP.

The different action of these thio-compounds could contribute in clarifying the mechanisms involved when they interfere with cis-DDP.

MATERIALS AND METHODS

The cell line used was F10/B16 metastatic melanoma cells (Fidler's source). The drugs used were : cisdiamminodichloroplatinum (cis-DDP) and 2,2'-dithiopyridine (2,2'-PDS) (Sigma Chemical Co.); 6,6'-dithiodinicotinic acid (CPDS) and 6-mercaptonicotinic acid (6-MNA) (synthesized by dr. D.R. Grassetti); sodium 2-mercaptoethanesulfonate (Mesna) (Schering SpA).

The drugs were diluted to the required concentrations with medium (DMEM : Flow) plus 10% foetal calf serum (Flow), 1% 200 mM

glutammine, 1% Hepes buffer (Flow), 100 U/ml penicillin, and 50 µl/ml kanamycin (Farmitalia).

Cell growth

The cells were exposed for 1 hr in suspension to 0.1 mM CPDS, to 0.03 mM and 0.003 mM cis-DDP and to the combinations of CPDS + cis-DDP.

The control cells were suspended for 1 hr in normal medium.

Then, the cells were centrifuged, seeded ($3x10^4$ cells/dish) and incubated in normal medium at 37° in 5% CO_2 atmosphere.

After 72 hr the cultures were trypsinized and counted in a haemocytometer. The percentage of growth inhibition of treated cells in comparison to the control ones was considered.

Cloning assay in liquid medium

The procedure used was identical to the previous one, but the cells exposed to the drug for 1 hr, were seeded at a density of $3x10^3$ cells/dish. After 4-5 days, when the colonies (more of 40 cells/colony) were visualized, they were stained by May Grünwald Giemsa and counted.

Multiwells screening test (Sauter's method)

F10 cells were seeded at a density of $8x10^4$ cells/well. When the monolayer was formed (after 24 or 48 hr) the cells were exposed to cis-DDP from 0.6 mM to 0.009 mM alone or in combination with 1.5 mM CPDS, 0.15 and 0.015 mM 2,2'-PDS, 3 mM Mesna, 0.2 mM 6-MNA. Some wells were treated with the thio-compounds alone at the reported concentrations.

After 24 hr the culture medium was removed. The cell monolayers were rinsed with saline solution and stained with methylen blue-parafuchsin (14). This staining procedure removed the dead cells from the wells. A complete cytopathogenic effect (CPE) was evaluated when all the cells were removed. The cytotoxicity of cis-DDP and the protection or the enhancement of this effect by

thio-compounds were considered.

Changes in cis-DDP minimal inhibitory concentration (M.I.C.) due to the combination with thio-compounds were reported.

RESULTS

Cell growth (Fig. 1)

0.1 mM CPDS was not toxic for cells and caused only a slight inhibition of cell growth (22%); 0.03 mM and 0.003 mM cis-DDP reduced cell growth, respectively to 85% and 52% of the controls; CPDS increased the effect of 0.03 mM cis-DDP (90% of inhibition) but had no effect on 0.003 mM cis-DDP (50%).

Cloning assay in liquid medium (Fig. 2)

The percentage of cloning efficiency of cis-DDP was concentration dependent : in 0.0003 mM cis-DDP and 0.15 mM CPDS treated cells the percentage was similar to the controls. In comparison with the controls the cloning efficiency in the presence of 0.03 mM cis-DDP was 77.7% and 58.9% when combined with CPDS, in the

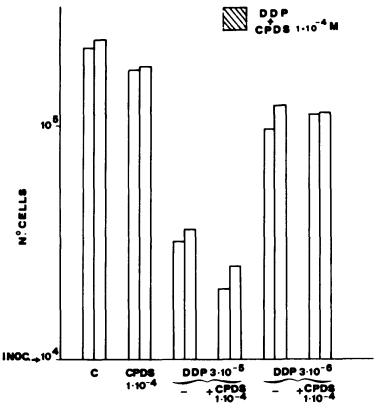

Fig. 1. Effect on cell growth of 1 hr exposure of CPDS, cis-DDP and CPDS/cis-DDP. Cell counting was carried out after 72 hr of growth. Representation of two experiments.

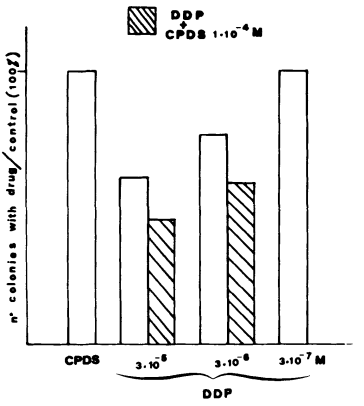

Fig. 2. Cloning assay. Percentage of cloning efficiency of drug treated cells in comparison to controls.

presence of 0.03 mM cis-DDP it was 61.1% and 46% when combined with CPDS.

Multiwells screening test (Fig. 3)

A concentration-dependent CPE of cis-DDP from 0.6 mM to 0.07 mM was demonstrated. CPDS, 6-MNA, 2,2'-PDS and Mesna at the concentrations indicated in Fig. 3 had no CPE.

The combinations of the thio-compounds with cis-DDP showed : Mesna antagonized the cis-DDP effect by enhancing cis-DDP M.I.C. (from 0.15 to 0.30 mM).

1.5 mM CPDS halved the cis-DDP M.I.C. to 0.07 mM. 2,2'-PDS had a stronger synergic action: in fact a ten fold lower concentration (0.15 mM) than CPDS was responsible for inducing much higher effect by reducing cis-DDP M.I.C. to <0.009 mM.

No change in cis-DDP M.I.C. was detected for 0.2 mM 6-MNA.

DISCUSSION

The different behaviour of Mesna in comparison with CPDS and 2,2'-PDS could be explained by the characteristics of the two

Thio-compound mM		M.I.C. cis-DDP mM	Effect
-		0.15	
CPDS	1.5	0.07	Synergism
2,2'-PDS	0.15	0.009	
2,2'-PDS	0.015	0.15	Indifference
6-MNA	0.2	0.15	
Mesna-Na	3	0.30	Antagonism

Fig. 3. Multiwells screening test. Minimal inhibitory concentration (M.I.C.) of cis-DDP alone or combined with several thio-compounds.

classes of compounds. Our results confirm the protective role exerted in vitro by Mesna.

CPDS is not toxic; in fact 0.1 mM CPDS (1 hr of treatment) does not inhibit colony formation or cell growth to a great extent (12) but 1.5 mM CPDS shows a synergic effect with cis-DDP following 24 hr treatment.

2,2'-PDS at a ten fold lower concentration shows a much stronger effect than CPDS : it could suggest that the site for synergic interaction is probably localized inside the cells; in fact the possibility of 2,2'-PDS in penetrating into the cell is higher than that of CPDS.

cis-DDP itself reduces glutathione (GSH) (an intracellular tripeptide for detoxification of xenobiotic compounds) in kidney and liver (15).

An increase in GSH content was shown in tumor cells resistant to antineoplastic drugs (16) : to be considered as a mechanism of self protection produced by the cells against cytotoxic drugs.

Treatment with buthionine sulfoximine (inhibitor of GSH synthesis) sensitizes some types of resistant and sensitive cell lines to the toxic effect of cis-DDP (17,18). CPDS and 2,2'-PDS are able to oxidize GSH : their synergic effect with cis-DDP could be explained by the reduction of GSH content in the cells. Cell membrane modification induced by CPDS by adding negative charges and demonstrated by electrophoresis analysis (10) and by a decrease in cell adhesiveness (12), could be an important factor in enhancing the cis-DDP penetration into the cells.

It was shown that certain analogs of nicotinamide inhibit the formation of poly adenilic acid (poly A) from NAD in the cell nucleus. Poly A is involved in DNA repair after damage caused by antineoplastic drugs such as streptozotocin or methyl nitrosureas occurs (19). Considering that CPDS and 2,2'-PDS are sulfur containing analogs of nicotinamide, we could assume that these drugs sensitize the cells to cis-DDP by means of inhibition of DNA repair.
A potentiation of some other antineoplastic drugs after treatment with poly A synthetase inhibitors (3-acetamido benzamide) was

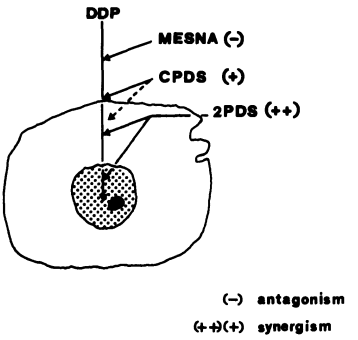

Fig. 4. Hypothetic sites of interaction of Mesna, CPDS and 2,2'-PDS with cis-DDP.

observed (19,20).

Therefore three possible mechanisms could be considered in order to explain such a synergic effect : 1) oxidation of GSH; 2) change of cell surface with an enhancement of cis-DDP penetration into the cells; 3) inhibition of poly adenilic acid formation from NAD (Fig. 4).

Studies might be carried out to evaluate if the observed in vitro synergic effect on tumor cell killing is also present in vivo; and whether it could be considered as an enhancement of cis-DDP systemic toxicity or be limited to the tumor cell population.

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A NEW CLASS OF HYPOXIC CYTOTOXINS [$PtCl_2$ (NH_3) (nitroimidazole)]

K.A. Skov, N.P. Farrell and D.J. Chaplin

At the previous ISPCC Conference (1), we described initial attempts to target nitroimidazoles and other hypoxic cell radiosensitizers (L) to DNA, the target of ionizing radiation, using platinum, which itself has been demonstrated to interact with radiation preferentially in hypoxic cells. These early compounds, $[PtCl_2L_2]$, are generally not better sensitizers than 2L (2,3,4). We have since found that the bis-nitroimidazole complexes do not appear to bind to DNA, when assessed by their inhibition of restriction endonuclease activity on plasmid DNA (5). Two exceptions have been identified which do bind to DNA, and which are better sensitizers than 2L (6,7). In addition, a mono series of the form $[PtCl_2(NH_3)L]$ has been produced and found to bind to DNA and to sensitize better than their bis analogues, $[PtCl_2L_2]$ (5,8,9). Nitroimidazoles used as L include misonidazole (a 2-nitroimidazole used clinically as a radiosensitizer), metronidazole (a 5-nitroimidazole used to combat anaerobic infections) and 4-nitroimidazole.

The toxic properties of the monoseries, suggesting chemotherapeutic potential, are also of interest:

1. While there is measurable toxicity in aerobic cells (presumably due to the DNA binding), the toxicity is much greater in hypoxic cells (presumably due to L, compounds which are generally toxic to hypoxic cells upon reduction) (7).
2. trans-$[PtCl_2(NH_3)$(misonidazole)] exhibits higher toxicity in aerobic or hypoxic cells than the cis isomer (7).
3. Those complexes examined to date in vivo show antitumour activity (KHT sarcoma, Lewis lung carcinoma).

4. This tumoricidal effect is greatly enhanced by the vasoactive drug, hydralazine, presumably by increasing the hypoxic fraction, and thus enhancing the activity of the hypoxic cytotoxin. RSU-1069, a nitroimidazole compound with an aziridine function to bind to DNA, also exhibits increased toxicity in these systems with vasoactive compounds (10,11).
5. Futhermore, the complex with L=metronidazole may be more active than that of L=misonidazole. This would not be predicted on the basis of the properties of free L.

Further studies on the chemotherapeutic potential of this new mono series are planned, including a more thorough study of the entire series in vivo, and mechanistic studies in vitro.

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CISPLATIN INTERACTIONS WITH REDUCED GLUTATHIONE: AN *IN VITRO* STUDY

S. Stefanelli, O. Faggionato, M. Peracchi, M. Carrara, L. Cima and M.V. Fiorentino

INTRODUCTION

Recently, the use of reducing thiols and reduced glutathione (GSH) has been proposed in vivo in an attempt to lower cis-platinum (DDP) induced nephrotoxicity. Thiol-containing compounds have been tested as antidotal agents, since toxic effects of DDP are reminiscent of heavy metal intoxication (1). It is well known that the nephrotoxicity of the heavy metals appears to be related to a depletion of intracellular GSH or attachment to SH groups of proteins necessary for enzyme function (2). Also the nephrotoxic effect of DDP may be related to depletion of SH groups.

We have studied the effects of reduced GSH on B16-F10 cells concurrently exposed to cytotoxic doses of DDP, for better elucidating drug-drug interactions in an in vitro system.

MATERIALS AND METHODS

Effect on cell growth.

The experiments were performed using F10 metastatic cells of B16 murine melanoma (Fidler's source). The cells were seeded at $3x10^4$ cells/ml in R.P.M.I. 1640 medium supplemented with 8% foetal calf serum (FCS Gibco), 1% 200 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Each experiment was set up in triplicate in Petri dishes. The cultures were exposed to compounds, incubated in an atmosphere containing 5% CO_2 at 37°C, recovered from culture dishes by 0.25% trypsin, neutralized by FCS, centrifuged at 1000 rpm for 10 min and counted in a hemocytometer. GSH, dissolved in saline and diluted in medium in order to evaluate the cell growth, was tested at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M after 24, 48, 72 hrs exposure. Cell survival was evaluated in the culture exposed to DDP 20 μg/ml (ID_{50}) or to GSH at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M for 1 hr, washed three times, incubated for 1.5 hrs with growth medium and then exposed to medium containing GSH (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M) or DDP 20 μg/ml for 1 hr (3). The cell count was performed after 24 hrs. All the results are reported as per cents of controls and were statistically evaluated by Student's t-test.

Assay for colony formation.

The basic agar procedure used was that of Hamburger and Salmon (4), with some variations. Briefly, an underlayer of 0.5% agar in enriched R.P.M.I. medium 1640 containing 10% FCS was prepared (3 ml in a 35 mm Falcon plastic Petri dish). Cells to be tested for colony formation were suspended in a plating layer of 0.3% agar in enriched R.P.M.I. 1640 medium with 25% FCS. The cells were exposed to DDP 1 μg/ml (ID_{50}) for 1 hr and pre-treated or post-treated with 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M GSH for 1 hr. Cells were plated at a concentration of 10^3 cells/ml in the 1 ml plating layer. Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere

of air. Cultures were examined with an inverted microscope at x 100 and scored 14 days after plating. Colonies were defined to be aggregates of more than 40 cells.

In vitro incorporation of ^{3}H-thymidine.

Cells were seeded in sterile plat-bottomed plastic plates (24 wells, diameter 16 mm, Costar, Cambridge, Maryland) in 1 ml R.P.M.I. 1640 medium supplemented with 8% FCS and grown in a 5% CO_2 atmosphere at 37°C. After 24 hrs of incubation, 1 ml of DDP or GSH diluted in medium was added at different concentrations (10^{-3}, 10^{-4}, 10^{-5}M) and incubated for 2 hrs. After this period 0.5 μCi of ^{3}H-thymidine (sp. act. 14 Ci/mmol, 1 mCi/ml) was added for another hour (5).

GSH pre-treatment. The cells were pre-treated for 1 hr with GSH (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M), washed, exposed to DDP 100, 200, 300 μg/ml for 1 hr, washed again and incubated for 1 hr or 24 hrs in medium. After this period 0.5 μCi of ^{3}H-thymidine was added for another hour.

GSH post-treatment. The cells were post-treated for 1 hr with GSH 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M, washed, exposed to DDP 100, 200, 300 μg/ml for 1 hr, washed again and incubated with ^{3}H-thymidine for 1 hr directly or after 24 hrs. Cells were washed twice in cold CMF-PBS, three times in 5% trichloroacetic acid, and once in ethanol. The dry trichloroacetic acid insoluble material was then dissolved in 1 ml 1N NaOH, neutralized with 1 ml 1N HCl, and counted in 10 ml Instagel (LKB Instruments).

The results are reported as pmoles/10^{6} cells.

RESULTS AND DISCUSSION

Effects on cell growth.

At 10^{-3}M GSH caused 25%, 50%, and 70% cell growth decrease after 24, 48, and 72 hrs exposure, respectively. No growth

inhibition was observed at lower concentrations. At 20 μg/ml DDP caused a 50% decrease of the viable cells (ID_{50}) after 1 hr exposure (Fig. 1).

The pre-treatment for 1 hr with non toxic concentrations of GSH (10^{-4}, 10^{-5}, 10^{-6}M) offered no significant protection against the ID_{50} of DDP.

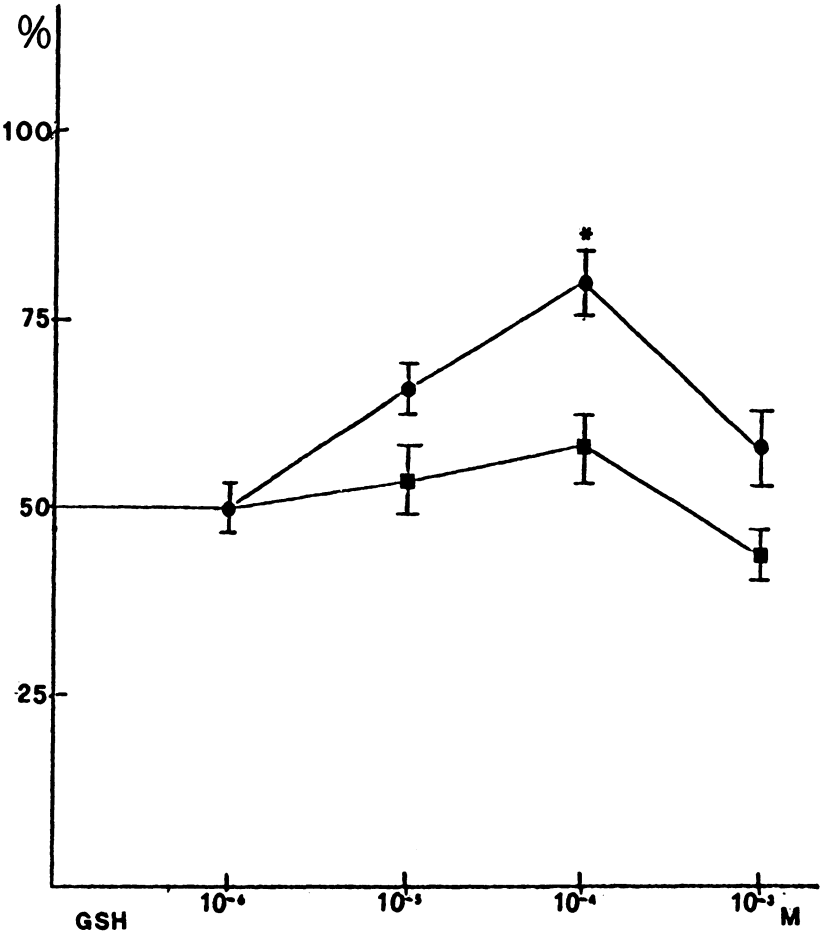

FIG. 1. Survival of F10 cells after 1 hr exposure to DDP 20 μg/ml (ID_{50}), and pre-treated (■) or post-treated (●) for 1 hr with GSH (*$p \leq 0.05$).

The post-treatment for 1 hr with the same non toxic concentrations of GSH caused a reduction of the DDP cytotoxic response which reached its maximum at 10^{-4}M ($p \leq 0.05$).

Colony formation.

GSH at 10^{-5}M and up to 10^{-3}M caused no significant changes of colony formation after 1 hr exposure. At 1 µg/ml DDP caused a 50% decrease of colony formation (ID_{50}) after 1 hr exposure (Fig. 2).

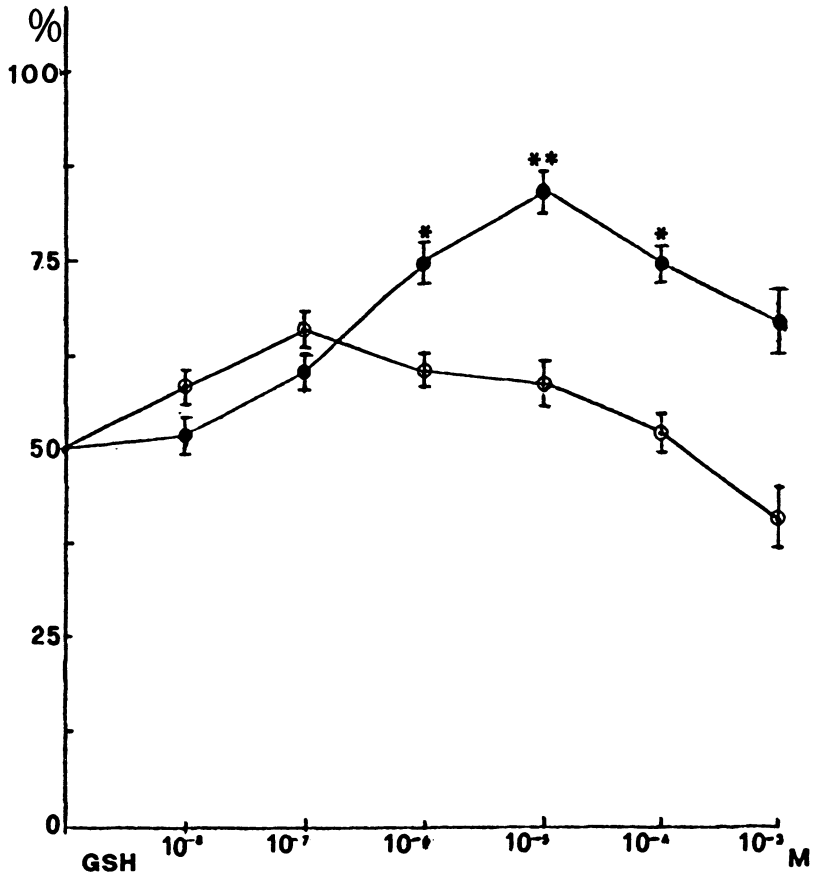

FIG. 2. Colony formation of F10 cells after 1 hr exposure to DDP 1 µg/ml (ID_{50}), and pre-treated (o) or post-treated (●) for 1 hr with GSH (*$p<0.05$; **$p<0.01$).

The pre-treatment for 1 hr with GSH at 10^{-8}M and up to 10^{-3}M offered no significant protection against the ID_{50} of DDP.

The post-treatment for 1 hr with GSH at 10^{-4}M and up to 10^{-6}M caused a significant decrease of the DDP cytotoxic response, which reached its maximum at 10^{-5} ($p<0.01$).

Incorporation of ^{3}H-thymidine.

GSH at 10^{-3}M and up to 10^{-5}M caused no significant change of the ^{3}H-thymidine incorporation after 3 hrs exposure, indicating that GSH does not affect the DNA synthesis. When DDP was tested at the same concentrations and exposure time, no reduction of the incorporation was observed except at 10^{-3}M (-95%).

In order to compare these results obtained on cell growth and colony formation, an exposure time of 1 hr for DDP was chosen also in the incorporation test. After exposure to ^{3}H-thymidine for 1 hr, its incorporation (pmoles/10^{6} cells, enclosed in brackets) 2 and 23 hrs later was reduced by 92% (0.23), 82% (0.55), 50% (1.5) and 96% (0.13), 91% (0.26), 88% (0.37) after 1 hr exposure to DDP at 300, 200 and 100 μg/ml, respectively.

The pre-treatment for 1 hr with GSH at 10^{-2}M and up to 10^{-6}M caused no significant changes of incorporation either 2 or 23 hrs following DDP. These results seem to confirm that GSH does not affect DNA synthesis, in accordance with the results obtained after 3 hrs exposure to GSH alone.

The post-treatment for 1 hr with GSH at 10^{-2}M and up to 10^{-6}M caused no significant change in the incorporation evaluated after 2 hrs of exposure to the three concentrations of DDP, while significant reductions were observed after 23 hrs exposure to 300, 200 and 100 μg/ml of DDP (Fig. 3).

These results indicate that GSH post-treatment, but not pre--treatment, induces a dose-dependent protection against DDP cytotoxicity and this effect increases with time, probably related to a

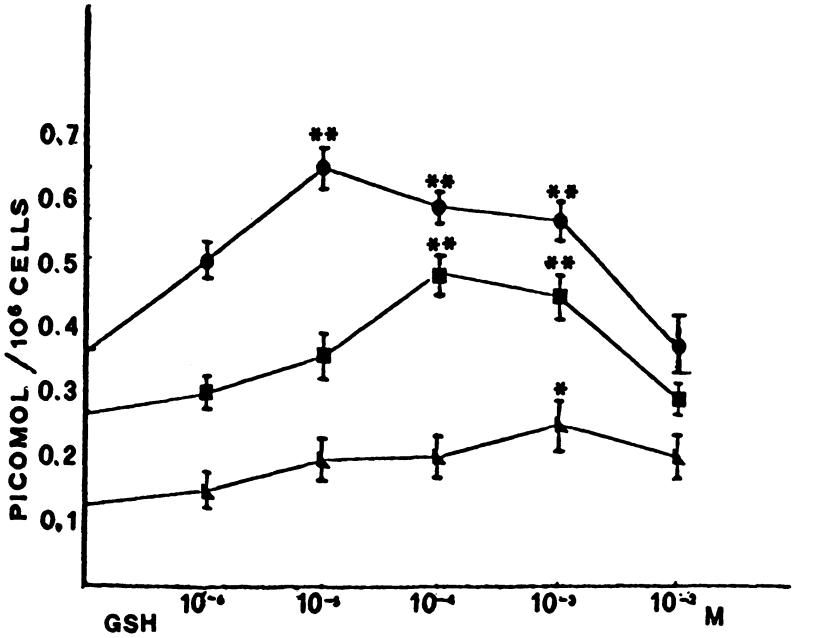

FIG. 3. In vitro incorporation of ^{3}H-thymidine by F10 cells evaluated 23 hrs after 1 hr exposure to DDP 300 (◣), 200 (■), and 100 (●) μg/ml followed by treatment with GSH (*p<0.05; **p<0.01).

further prevention of the cell damage induced by the antitumor agent. In fact, the maximum values of pmoles/10^{6} cells (enclosed in brackets) of ^{3}H-thymidine after 23 hrs were about 92% (0.25), 85% (0.48), and 75% (0.65) higher than following the exposure to 300, 200 and 100 μg/ml of DDP alone.

In conclusion, all the three tests employed show that there is a drug-drug interaction in the treatment sequence DDP-GSH, but not in the treatment sequence GSH-DDP. Moreover, the incorporation test shows that the longer is the time from GSH post-treatment at which the thymidine test is performed, the more evident is the prevention of DDP cytotoxicity by GSH. Studies are in progress to clarify if this response is due to interaction of GSH with DDP

metabolites and consequent formation of Pt(II)-adducts possibly less cytotoxic than DDP.

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DETERMINATION OF PLATINUM AND BIOLOGICALLY ESSENTIAL TRACE ELEMENTS BY NEUTRON ACTIVATION ANALYSIS IN PHARMACOKINETIC STUDIES ON CISPLATIN DERIVATIVES WITH ESTROPHILIC LIGANDS

S. Trebert Haeberlin, F. Lux, T. Spruss, R. Gust and H. Schönenberger

INTRODUCTION

Cis-diamminedichloroplatin(II) (Cis) is one of the most important cytostatic agents being used for chemotherapy of various neoplasms. Unfortunately, Cis has only a small tumor-inhibiting effect on the hormone-dependent mammary carcinoma. It is known that estrogens are enriched in the estrogen receptor containing mammary tumor cells and have in higher concentrations a certain tumor-inhibiting effect. The antitumor activity and the estrophilic properties of substituted 1,2-diphenylethylenediamines (1, 2, 3, 4) gave rise to the idea of using such compounds as neutral ligands with carrier function for cisplatin derivatives. It was supposed that such platinum compounds would be enriched in estrogen receptor positive tissues like the mammary carcinoma and would show a strong inhibitory effect on mammary tumors. Ring-substituted [1,2-bis(4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II)-complexes were synthesized and then tested by special in vitro/in vivo-screening procedures, which confirmed the antitumor activity and the estrophilic behaviour of the respective compounds (5, 6). Unfortunately, these complexes have a low solubility in water, so that they have to be administered as suspension in oil or dissolved in a 1:1-mixture of polyethyleneglycol and 0.9 % aqueous sodium chloride solution (PEG 50). The results of the in vitro- and in vivo-experiments with meso-dichloro-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamineplatinum(II) = A are reported in (5, 6, 7). In the meantime, the water soluble drug meso-diaqua-1,2-bis(2,6-dichloro-4-hy-

droxyphenyl)ethylenediamineplatinum(II)-sulfate = A' was synthesized (8) and administered in aqueous solution to Sprague-Dawley-rats bearing DMBA-induced hormone-dependent mammary carcinoma (SD-rats, DMBA).

Neutron activation analysis (NAA) was applied to provide information on

(i) the distibution of the used drug in the organism by means of the determination of the platinum contents (EC(Pt)) of tumor tissue, blood and different organs, and

(ii) changes of the trace element balance during carcinogenesis and under the influence of the therapy by means of the determination of the biologically essential trace elements' contents (EC(El)) of tumor tissue and different organs of therapied and not therapied SD-rats, DMBA.

MATERIALS AND METHODS

The NAA procedure is described in detail in (7). Fig. 1 shows a flow sheet of the chemical separation being used for multi-element-determination.

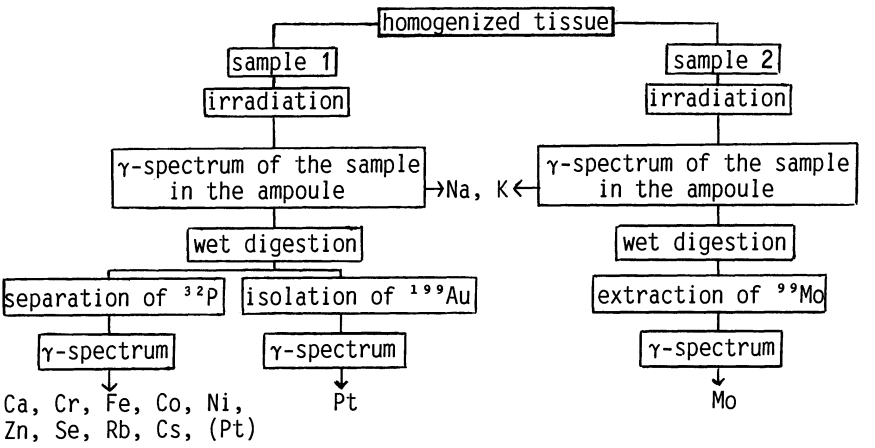

Fig. 1. NAA procedure for multi-element-determination

Tab. 1. Comparison of the efficiency of NAA and ICP

ICP: inductively coupled plasma emission spectroscopy
m(El): mass of the element found in the analysed sample
$m(El)_C$: detection limit. In NAA $m(El)_C$ is the mass of the element corresponding to L_C (Critical Level, (9)). The $m(El)_C$ values of ICP are taken from (10).

Element	$m(El)_C$ NAA ng	$m(El)_C$ ICP* ng	$m(El)_{tumor\ sample}$ (150 mg fresh weight) ng
Na	200	29	193 000
K	18 000	428 570	340 000
Ca	2 800	5.5	10 000
Cr	0.018 0.11**	60	< 1
Fe	113	25	4 700
Co	0.15	78	0.38
Ni	50	100	< 50
Zn	12	34	1 800
Se	1	750	30
Mo	0.2	93	10
Pt	0.24	300	63

* 10 ml solution
** blank value for chromium (11)

The data in Tab. 1 show the necessity of the use of NAA in the investigations: In a typical tumor sample only 4 of the listed 11 elements can be determined by ICP, but 9 by NAA.

Animal experiments

I. SD-rats, not tumor-bearing

I.1 i. p. single application, A in PEG 50
dose*: $1.5 \cdot 10^{-5}$ mol/kg ≙ 3.0 mg Pt/kg

I.2 s. c. single application, A in PEG 50
dose*: $1.0 \cdot 10^{-5}$ mol/kg ≙ 1.95 mg Pt/kg

II. SD-rats, DMBA; 4 weeks' therapy, s. c. application 3x per week

II.1 controls (Ko), PEG 50

II.2 ligand of A respectively A' in PEG 50 (Lig)
total dose*: $6.0 \cdot 10^{-5}$ mol/kg

II.3 A in PEG 50 (A)
total dose*: $12 \cdot 10^{-5}$ mol/kg ≙ 23.4 mg Pt/kg

II.4 A' in H_2O (A')
total dose*: $6.0 \cdot 10^{-5}$ mol/kg ≙ 11.7 mg Pt/kg

II.5 Cis in PEG 50 (Cis)

total dose*: $6.0 \cdot 10^{-5}$ mol/kg ≙ 11.7 mg Pt/kg

* dose of the drug corresponding to 1 kg body weight

RESULTS AND DISCUSSION

Platinum

The platinum blood level was chosen as indicator for that part of the administered dose of the drug being resorbed by the individual animal (experiment I). Fig 2 shows the course of $EC(Pt)_{blood}$ up to 72 h after the application of A. Only a small portion of the administered dose of platinum was found in the blood: 1 % (corresponding to the 12 h value of Fig. 2) after s. c. application, 9 % (corresponding to the 6 h value of Fig. 2) after i. p. application. These data are in accordance with the reported excretion of 80 - 90 % of the applied platinum (12, 13).

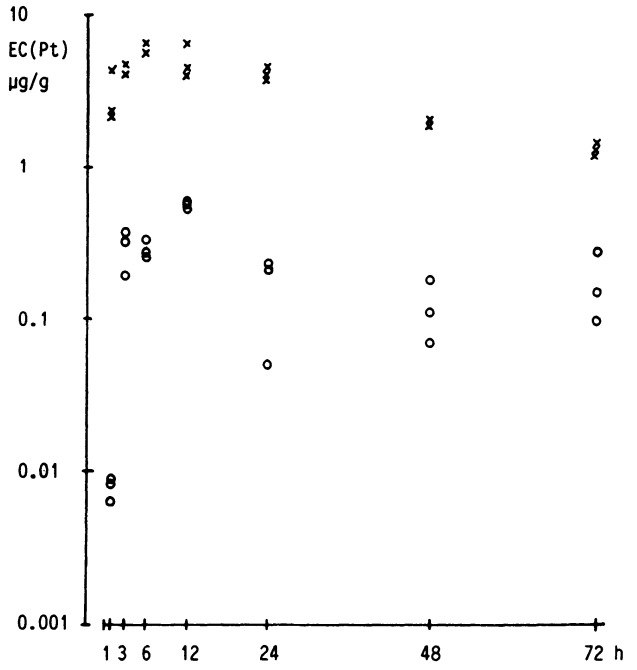

Fig. 2. Platinum blood level of SD-rats after single application of A in PEG 50, influence of the way of application (i. p. and s. c., resp.)
x: i. p., D(Pt) = 2.9 mg/kg; o: s. c., D(Pt) = 1.95 mg/kg
EC(Pt) = content of platinum
D(Pt) = dose of platinum corresponding to 1 kg body weight
(experiments I.1 and I.2)

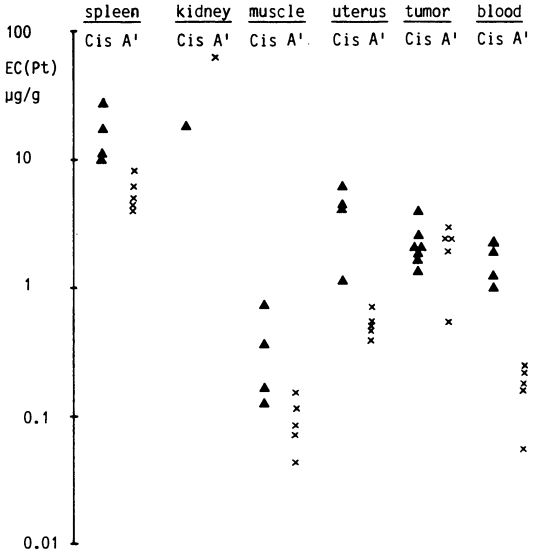

Fig. 3. Contents of platinum (EC(Pt))
SD-rats, DMBA, application s. c., day 28 of therapy
Cis: dissolved in PEG 50, D(Pt) = 11.7 mg/kg
A': dissolved in H_2O, D(Pt) = 11.7 mg/kg
D(Pt) = dose of platinum corresponding to 1 kg body weight
(experiments II.4 and II.5)

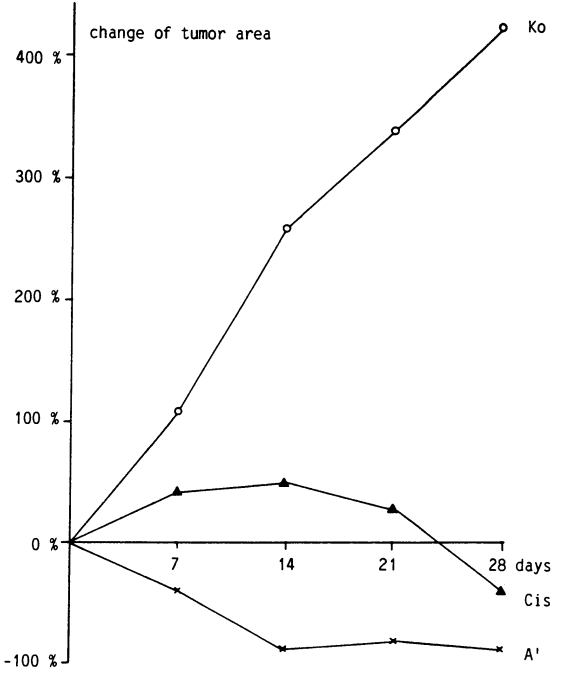

Fig. 4. Change of tumor area in dependence of the duration of the therapy (4 weeks); application 3x per week, s. c.
Ko: control, PEG 50
Cis: dissolved in PEG 50, D(Pt) = 11.7 mg/kg
A': dissolved in H_2O, D(Pt) = 11.7 mg/kg
D(Pt) = dose of platinum corresponding to 1 kg body weight
(experiments II.1, II.4 and II.5)

In Fig. 3 the results obtained in the determination of platinum (experiments II.4 and II.5) are presented. The $EC(Pt)_{tumor}$ were more or less the same using A' or Cis, but the $EC(Pt)_{blood}$ of the animals therapied with A' were only 1/10 of those therapied with Cis. Comparing the $EC(Pt)_{tumor}$ with $EC(Pt)_{muscle}$, i. e. with the EC(Pt) of a tissue with low estrogen receptor level, the ratio $\overline{EC}(Pt)_{tumor}$ to $\overline{EC}(Pt)_{muscle}$ = 22 after administration of A', but is only 6.2 after administration of Cis. From an analogous comparison with the EC(Pt) of the estrogen receptor-rich uteri results $\overline{EC}(Pt)_{tumor}$ to $\overline{EC}(Pt)_{uteri}$ = = 3.9 (A') and 0.53 (Cis), respectively. All the mentioned data indicate that A' has the expected high affinity to the hormone-dependent mammary carcinoma. Fig. 4 shows that, although the $EC(Pt)_{tumor}$ using A' or Cis are very similar (see Fig. 3), the antitumor activity of A' towards the receptor-positive mammary carcinoma is considerably higher than that of Cis.

The platinum-load of the spleen is lower using A' than using Cis. A statement about the load of the kidney requires more data, but in former investigations with mice the highest EC(Pt) values were obtained for the kidneys (7, 14).

Biologically essential trace elements

An enrichment or depletion of the respective trace element in a tissue is only regarded to be unequivocal, if

$\overline{EC}(El)_{therapied\ animal} \geq 3\ \overline{EC}(El)_{not\ therapied\ animal}$ or

$\overline{EC}(El)_{therapied\ animal} \leq 1/3\ \overline{EC}(El)_{not\ therapied\ animal}$.

Such results were only obtained for the contents of calcium, iron and molybdenum.

Calcium: According to Fig. 5, the $EC(Ca)_{tumor}$ of the animals treated with Lig or Cis differ hardly from those of the controls. In the case of the administration of A', however, high and strongly scattering EC(Ca) were obtained. A biochemical interpretation can not yet be given.

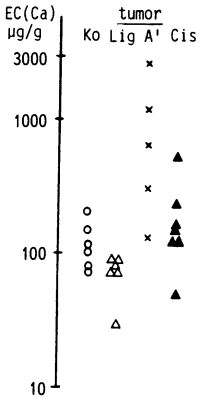

Fig. 5. Contents of calcium (EC(Ca))
SD-rats, DMBA, application s. c.
day 28 of therapy
Ko: control, PEG 50
Lig: dissolved in PEG 50
A': dissolved in H_2O, D(Pt) = 11.7 mg/kg
Cis: dissolved in PEG 50, D(Pt) = 11.7 mg/kg
D(Pt) = dose of platinum corresponding to
1 kg body weight
(experiments II.1, II.2, II.4 and II.5)

Iron: The data in Fig. 6 show clearly an influence of the therapy on the iron metabolism. The $EC(Fe)_{spleen}$ of therapied animals are very high, and that in the case of treatment with Lig, A' and Cis. The spleen plays an important role in the metabolic processes, especially in the degradation of erythrocytes and hemoglobin, the formation of bilirubin, the storage of the released iron, and in the phagocytosis of impurities of blood (15). On the other hand it is known that platinum compounds damage the bone marrow. It can be concluded that the iron enrichment in the spleen is a consequence of an increased degradation of damaged erythrocytes and the subsequent storage of iron.

The iron levels of tumors of therapied animals are slightly raised. However, the significance of these enrichments has to be proved in further investigations.

Molybdenum: Molybdenum is considerably enriched in tumors of animals therapied with A or A' (experiments II.3 and II.4) towards the tumors of controls (experiment II.1) and of those animals treated with Lig or Cis (experiments II.2 and II.5). Mammary glands of not tumor-bear-

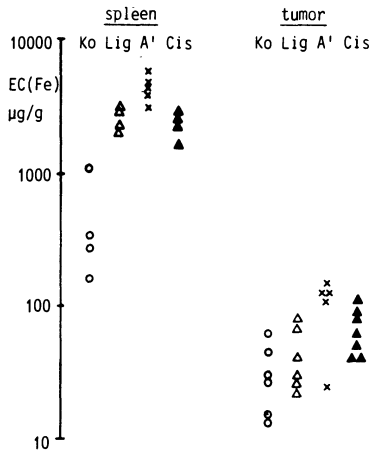

Fig. 6. Contents of iron (EC(Fe))

SD-rats, DMBA, application s. c., day 28 of therapy

Ko: control, PEG 50
Lig: dissolved in PEG 50
A': dissolved in H_2O, D(Pt) = 11.7 mg/kg
Cis: dissolved in PEG 50, D(Pt) = 11.7 mg/kg
D(Pt) = dose of platinum corresponding to 1 kg body weight

(experiments II.1, II.2, II.3 and II.4)

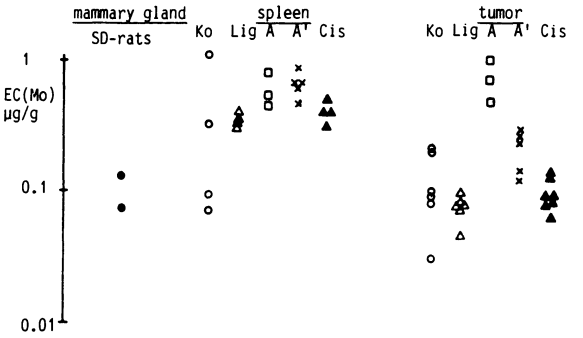

Fig. 7. Contents of molybdenum (EC(Mo))

SD-rats, DMBA, application s. c., day 28 of therapy

Ko: control, PEG 50
Lig: dissolved in PEG 50
A: dissolved in PEG 50, D(Pt) = 23.4 mg/kg
A': dissolved in H_2O, D(Pt) = 11.7 mg/kg
Cis: dissolved in PEG 50, D(Pt) = 11.7 mg/kg
D(Pt) = dose of platinum corresponding to 1 kg body weight

(experiment II)

ing SD-rats were analysed to get information on the molybdenum status of an unaffected comparative tissue. An enrichment by a factor of 2 (A') and of 6 (A) towards the mammary glands is to be recognized. A definite biochemical interpretation of these data can not yet be given, but it is assumed that some molybdoenzymes participate in the degradation of the tumors.

The $EC(Mo)_{spleen}$ of the therapied animals seem to be raised, but the $EC(Mo)_{spleen}$ of the controls scatter strongly so that an interpretation would not be admitted.

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SYNTHESIS AND THERAPEUTIC EFFECT OF NEW *CIS*-PLATINUM COMPLEXES ON EXPERIMENTAL TUMORS

R. Voegeli, J. Pohl, P. Hilgard, J. Engel, W. Schumacher, H. Brunner, M. Schmidt, U. Holzinger and H. Schönenberger

INTRODUCTION

The substitution of the two NH_3 groups in cisplatinum (cDDP) by ethylene diamine ligands leads to complexes with good pharmacological activity against experimental tumors (1). Our aim was to increase the activity/toxicity ratio and also to provide compounds against tumors which are either nonresponsive or resistant to cisplatinum. We prepared new platinum complexes of 1,2-diaminoethanes derived from phenylalanine.

CHEMISTRY

The substituted benzylethylenediamines 1 and 2 were prepared from the corresponding D,L-phenylalanine derivatives (Fig. 1).

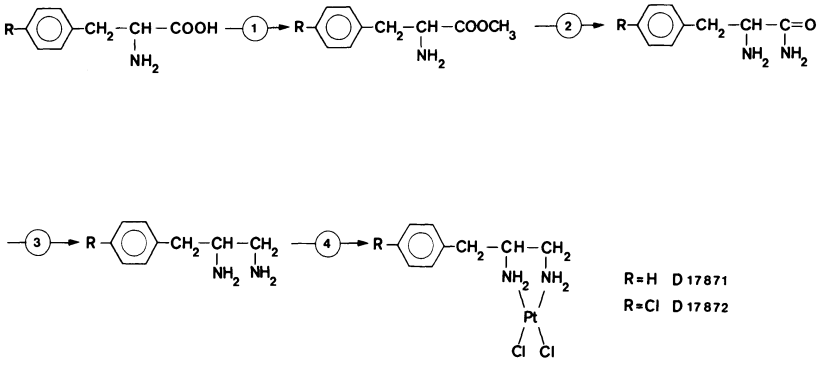

Fig. 1. Synthesis of D17871 and D17872

In step 1 the amino acid was converted to the methyl ester hydrochloride by reaction with thionyl chloride in methanol. The hydrochloride reacts with ammonia in methanol to give the amide (step 2). Reduction of the carbonyl group with $LiAlH_4$ in dry tetrahydrofuran gave the diamine (step 3).

The diamines 1 and 2 were used as ligands to prepare the dichloroplatinum (II) complexes. To an aqueous solution of K_2PtCl_4 the diamine was added. The complex (D 17871, R = H; D 17872, R = Cl) precipitated after a short time as yellow solid which was filtered, successively washed with water and methanol and dried in vacuum. The overall yields from the amino acids to the complexes were > 60 %.

The ligands and platinum complexes were characterized by their FAB-mass and ^{1}H-NMR spectra.

PHARMACOLOGY

The acute toxicity of the substances was determined by i.p. treatment of CD2F1 mice. The LD50 was 70 mg/kg for D17871 and 460 mg/kg for D17872

Rats treated with D17871 or D17872 showed no increase in the urea concentration in blood, whereas in rats treated with cDDP the concentration rises sharply as an effect of the nephrotoxicity of this compound (Fig. 2).

The substances were active in vitro against L1210 cells cultivated in soft agar. Concentrations resulting in 90% decrease in colony formation were 0.19 and 0.17 µg/ml for D17871 and D17872, respectively (Fig. 3).

Treatment of the P388 leukemia of the mouse with D17871 or D17872 in vivo resulted in a much greater increase in life span (136% and 132%, respectively) than treatment with cDDP (55%) (Fig. 4).

D17872 showed good efficacy in the treatment of the L1210 leukemia of the mouse (Fig. 5).

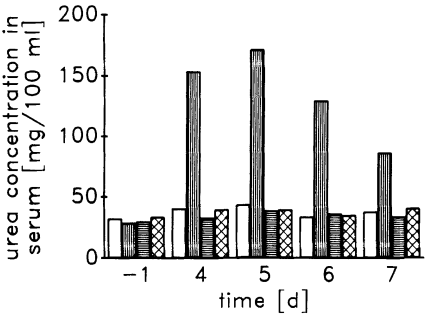

Fig. 2. Urea concentration in blood
Rats were treated i.p (cDDP i.v.), blood samples were taken the day before application, and from day 4 to day 7 after application. Urea was determined by measurement of urea nitrogen. (☐ control, ▥ cDDP, ▤ D17871, ▩ D17872)

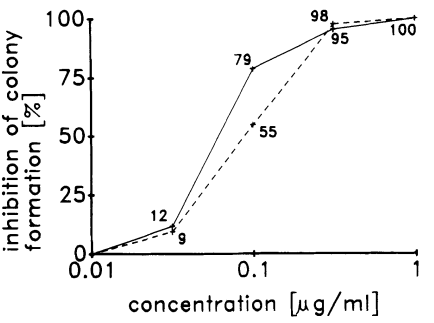

Fig. 3. Treatment of L1210 cells in vitro
Cells were cultivated in RPMI-medium with 10% fetal calf serum in 0.3% Agar. Solutions of test substances were placed on top. Cultures were incubated for 6 days and then colonies were counted (-- D17871, —— D17872)

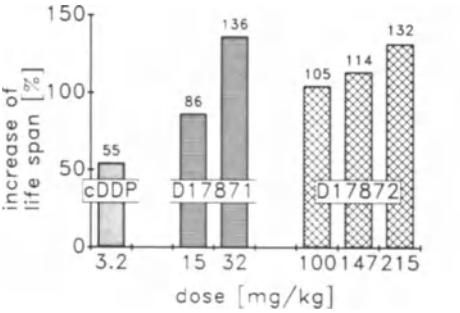

Fig. 4. Treatment of murine leukemia P388
Male CD2F1 mice were inoculated with 10^6 tumor cells i.p on day 0, test substances were applied i.p. on day 1.

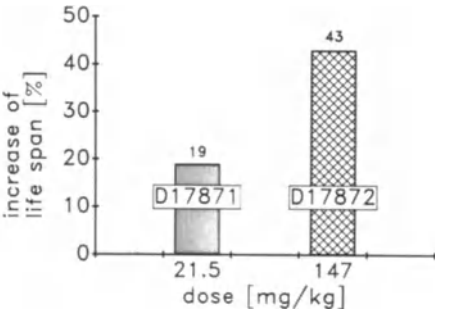

Fig. 5. Treatment of murine leukemia L1210
Male CD2F1 mice were inoculated with 10^5 tumor cells i.p. on day 0, test substances were applied i.p. on day 1.

Treatment of female C57B16 mice inoculated with $5*10^5$ B16 melanoma cells with D17872 (6.8 mg/kg/d, d1-6) resulted in a decrease of tumour mass by 49% relative to tumors of untreated animals (tumor weight was assessed 21 days after tumor inoculation).

Treatment of the M5076 reticulum cell sarcoma implanted into the subrenal capsule with D17872 inhibited tumor growth totally (Fig. 6).

Dimethylbenz(a)anthracene (DMBA) induced mammary tumors of rats were influenced favourably by daily treatment with D17872. cDDP was only of slight effect (Fig. 7).

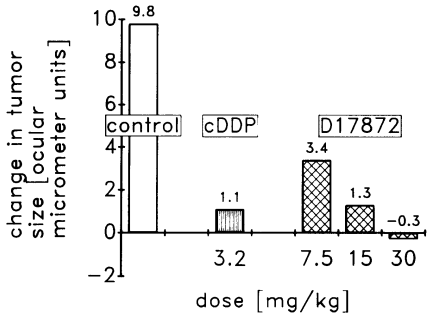

Fig. 6. Treatment of M5076 sarcoma
Tumor was implanted in B6C3F1 mice under the renal capsule on day 0. Mice were treated with test substances daily from day 1 to 5. Animals were killed on day 6. Tumor size was measured day 0 and day 6.

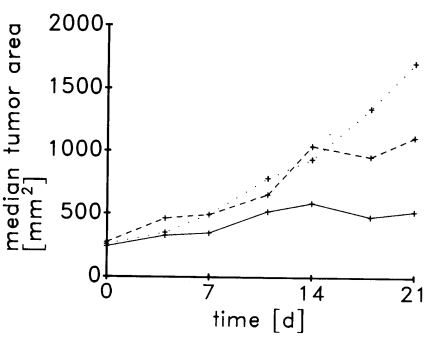

Fig. 7. Treatment of DMBA-induced mammary tumor
Female Sprague-Dawley rats bearing mammary tumors after induction with DMBA were treated five times per week (D17872 i.p., cDDP i.v.). Tumor areas were day measured twice weekly (... control, -- cDDP, —D17872)

CONCLUSION

D17871 and especially D17872 have shown good efficacy in the treatment of various animal tumor models. They compare favourably with cisplatinum. In addition to the better results achieved in these tumor models, both new substances are far less nephrotoxic than cDDP, as shown by the effect on the blood urea concentration.

Further study of compounds of this class is under way and should lead to substances with good efficacy and low toxicity for the treatment of human malignant disorders.

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C: Clinical Applications of Platinum Complexes.

THE DEVELOPMENT OF MORE EFFECTIVE PLATINUM THERAPY

I.H. Krakoff

The contributions of Barnett Rosenberg to the development of cisplatin as an effective antitumor agent have been abundantly acknowledged in this symposium and elsewhere. It is of historical interest that soon after he and his colleagues reported the evidence that this compound and others had promising activity in preclinical systems, Speer, et.al. initiated clinical trials and presented the very preliminary results in Prague in August, 1971. Two years later it was clear that an important new group of compounds had been discovered; the chemical and biologic information was reviewed at the Second International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy at Oxford in April, 1973. At that meeting, the clinical trials to date were also summarized. Hill summarized the sense of that meeting, stating that this group of compounds was too toxic for general use but therapeutically too effective to throw away. Mixed with the sense of enthusiasm for all of the new information and concepts was a sense of frustration.

The Third International Symposium was held in October, 1976, in Dallas. By that time there had been accelerating interest in the platinum

compounds and unequivocal evidence of useful therapeutic activity. Also by that time it was obvious that platinum toxicity, though important, was manageable.

A fourth symposium was held in Burlington in June, 1983, and this symposium is the fifth to be formally designated an international symposium in the same series. There has, of course, been marked interest in the development of the platinum compounds in cancer chemotherapy with numerous local, regional and national meetings and sections of other congresses devoted to studies of the advances in the therapeutic use of the platinum coordination complexes. In the spring meetings of the American Society of Clinical Oncology and the American Association for Cancer Research held in Atlanta this spring, hundreds of abstracts dealt with these compounds.

The major therapeutic activity has been clearly defined; cisplatin is of major importance in the treatment of testicular, ovarian and head and neck cancers, and an important adjunct in cancers of the bladder, cervix and lung. With the introduction of additional therapeutic regimens it is likely that other tumors will fall within the therapeutic spectrum of this group of drugs. Several of the papers at this symposium deal with new and different therapeutic attempts.

The toxicity of cisplatin has also been well defined. Renal toxicity continues to be a major problem although no longer prohibitive. Similarly, cisplatin is among the most emetic compounds used in cancer chemotherapy, although the judicious use of antiemetics has modified that distressing side effect. Cisplatin has produced only minor

hematologic toxicity, although with the elimination of nephrotoxicity as a limiting factor, some of the newer compounds have demonstrated significant myelosuppression. Direct toxicity to the gastrointestinal epithelium has been minimal.

The nephrotoxicity which was so severe that in 1973 it was questionable that this group of compounds would ever achieve general therapeutic use, has been approached effectively through regimens of hydration and diuresis, and through chloride loading.

No effective mechanism for preventing ototoxicity has been developed. Indeed, with the decreasing prominence of nephrotoxicity as a limiting factor, a variety of effects on the central nervous system have become much more prominent.

In attempts to extend further the utility of this group of compounds there have been major efforts to develop new, less toxic, or more effective analogs. Those efforts have been successful to a limited extent. Carboplatin and Chip are both in widespread clinical trial; both compounds appear to be non-nephrotoxic and less emetic than cisplatin and both have demonstrated antitumor activity. It is not yet certain whether these compounds are therapeutically more or less effective or equal to cisplatin.

It is encouraging that a group of "third generation" platinum compounds is presently being studied and may soon enter clinical trial. Among the papers to be presented at this symposium are several describing a new group of diaminocyclohexane compounds which are highly active and not cross resistant with cisplatin. Structural modifications have made those compounds highly soluble and,

in animal studies to date, completely free of nephrotoxicity. New delivery systems are also producing an impact with these third generation compounds. The incorporation into liposomes of several lipophilic platinum compounds has produced increased activity and negligible toxicity in preclinical studies. Early evaluation in patients with advanced cancer is anticipated.

Other studies to be reported in this symposium will include the intra-arterial delivery of cisplatin. It is now clear that the increase in local concentration which can be achieved with intra-arterial delivery has produced responses in hepatic metastases and has been a major element in achieving high cure rates with limb salvage in osteosarcoma of the extremities.

A major factor in our ability to use cisplatin effectively has been its incorporation into combination chemotherapy regimens. It has been noted that the use of compounds with different mechanisms of action and different target organs for toxicity may permit the utilization of two or more compounds, each at its maximum tolerated dose. That principle has permitted maximum exploitation of the therapeutic potential of cisplatin and has made it a major contribution in cancer chemotherapy.

As one looks back at the experience with this group of compounds during the last fifteen years, it is obvious that much has been achieved. It is more tempting, however, to look forward to the further development of this exciting group of therapeutic agents with the prospect for further major advance in our ability to treat a large and diverse group of tumors.

PLATINUM RADIOSENSITIZERS

D.H. Picker

Cisplatin is a well accepted agent used singly or in combination with other antitumor agents to treat a variety of human tumors, however, its use in combination with radiation is only now gaining wider attention.

In the radiotherapy community, it is generally accepted that the high rates of relapse and tumor radioresistance with radiation treatment are due to the existence of hypoxic (deficient in oxygen) regions in tumors and damage repair mechanisms.

Tumors are expected to contain significant populations of viable hypoxic cells, since oxygen is consumed before it penetrates distances greater than about 100 microns from the blood vessels. Radiation therapy is known to be less effective when cells are hypoxic as exemplified by the survival curve shown in Fig. 1. (1) From this standard in vitro survival curve, it is clear that radiation induced cell killing results in a steeper killing curve if oxygen is present. The cell killing factor Do (inverse of the slope of the exponential region of the survival curve) for aerated and hypoxic curves differ by approximately 3.0 and this enhancement due to the presence of oxygen is called the oxygen enhancement ratio (OER).

Similarly, enhancement of the cell killing efficiency in hypoxic cells when some other compound is present is called the sensitizer enhancement ratio (SER).

A major radio-biological research goal has been to overcome this radioresistance of tumor cells which might account for some of the cancer treatment failures. One approach has been to identify chemicals which are oxidizing agents that operate as radiosensitizers of hypoxic cells through free radical events that occur during irradiation. (2) A therapeutic gain might be expected if hypoxic cells in tumors are radiosensitized without any enhancing effect on aerated cells, assuming that most normal tissues are well oxygenated. Classes of electron affinic agents such as nitroimidazoles were identified and some of these compounds such as misonidazole were introduced into clinical trials for testing as radiosensitizers. Although a significant SER was demonstrated in cultured cells and in transplanted animal tumors, misonidazole proved to be neurotoxic. (3) Second generation compounds of this class have been developed and are currently undergoing clinical testing.

Discovery of Cisplatin as Radiosensitizer

The initial evidence for the interaction of radiation with cisplatin was published by Zak and Drobnik in 1971. They reported an increase in mouse survival when CP was given to leukemic mice concurrently with whole body radiation. As the primary target in the cell for both agents is assumed to be chromosomal DNA, an interaction between them leading to a greater than additive cell killing was suggested. This initial report has lead to a large number of studies in this area.

A. Results in Bacteria

In 1976 Richmond and Powers (5) found that the irradiation of bacterial spores in aqueous suspension in the presence of cisplatin resulted in survival curves showing pronounced

increases in radiation sensitivity in N_2 and O_2. Further studies with E. Coli found that irradiation with cisplatin also produced sensitization in N_2 saturated suspensions which was about 60% greater than that obtained in O_2 saturated suspension.

B. Mammalian Cell Studies

A large number of studies (6) have been carried out with mammalian cells with several investigators finding, after correction for cell killing by the drug alone, a true radiosensitizing effect with an average sensitizer enhancement ratio (SER) of 1.3. Sometimes, a reduction or total removal of the initial shoulder of the radiation dose response curve was observed, either as the only effect or together with an increase in the final slope of the curve.

One representative example is provided by the results from Douple in V79 cells (Fig. 2). (7)

In Vivo Studies

The results in animals are more difficult to analyze and vary substantially however, several investigators have found convincing evidence for supra-additive effects on murine tumors. (6) Douple et al. (8) used the tumor growth delay assay of a mouse mammary carcinoma (MTG-B) to assess the combination of CP and radiation. They found marked effects on tumor growth delay that were greater than additive with greater potentiation occurring when CP is given 1 hr before irradiation compared to immediately following radiation (Fig. 3) The fact that CP given after radiation treatment is effective raises the question of what the mechanism of this effect might be.

Mechanism

The lethal event to the cell caused by radiation is generally accepted to occur at DNA. Radiation may interact with water, which makes up about 80% of the cell's composition, to produce

hydroxyl radicals which are able to diffuse far enough to reach and damage this critical target. This is called indirect action of radiation and is considered to be the more important mechanism. (9) Alternatively the atoms of the target DNA may be ionized or excited initiating the chain of events that leads to lethal damage. This is called direct action of radiation. (9)

Oxygen acts as a radiosensitizer by interacting with this free radical damage on DNA and "fixing" it so that it can't be repaired.

The class of electron affinic nitroimidazole radiosensitizers, such as misonidazole, act as oxygen mimics by also interacting and "fixing" this free radical damage. (10)

When platinum complexes are present during radiation treatment, there are at least three mechanisms that might be responsible for the observed hypoxic sensitization: (6)

1) CP may fix the radical damage on DNA by acting as an oxygen mimic.

2) The platinum complex itself may interact with radiation or a product of water radiolysis to form a very reactive species that can cause the lethal event.

$(NH_3)_2Pt(II)Cl_2 \xrightarrow{e^-}{}_{(aq.)} (NH_3)_2Pt(I)Cl_2$

$(NH_3)_2Pt(II)Cl \xrightarrow{\cdot OH} (NH_3)_2Pt(III)Cl_2$

3) A Platinum - DNA mono adduct could react with radiation directly or a radiolysis product to achieve lethality.

The in vivo results using CP after radiation treatment to produce an enhanced cell kill relative to the additive effects expected from the two agents require additional mechanisms.

This potentiation of cell kill may result from molecular mechanisms such as the inhibition of repair of radiation damage by the platinum complex. A classic response to radiation is the cellular recovery when plateau phase cells are irradiated and

then incubated for several time periods prior to trypsinisation and subculture for viability assay. This effect is illustrated in Fig. 4 and is called (PLDR) potential lethal damage repair.(1)

When CP is added directly after irradiation, the PLDR assay demonstrates (Fig. 4) that CP inhibits the repair of lethal damage. Research with other Pt complexes has shown that other compounds are even more efficient at this inhibition with some compounds reducing the survival to less than expected by total inhibition of PLDR, an effect called enhanced chemotoxicity.(1)

Researchers have investigated a variety of other Pt complexes to gain further evidence about the critical mechanism. A variety of authors have found many Pt complexes that exhibit hypoxic sensitization[11] and many that are lacking in activity (Table 1). It is interesting to note that there is no correlation between antitumor activity and hypoxic sensitization.

Table 1

	Sensitization	
Compound	Oxic	Hypoxic
Cisplatin	+	+
trans-platin	0	+
Iproplatin	0	+
Carboplatin	0	+
Pt-dac-H_2O-SO_4	0	0
Pt-diaminoethane-malonate	0	0

Further in vivo results with carboplatin (12) have shown a better

tumor growth delay when the drug was given immediately after irradiation.

The message to be derived from this data is that the mechanism of interaction between Pt and radiation is complex and may even differ among Pt complexes.

The results of dose timing studies undertaken with CP and various Pt analogs suggest that the Pt complex must be present in free solution rather than bound to cellular components to be most effective. Since the pharmacokinetics differs for each compound, it becomes crucial to determine the time when the peak concentration of the appropriate species is present before conducting in vivo studies or carrying out clinical trials.

Previous pharmacokinetic studies (13) of ultrafilterable platinum levels in humans after IV infusion of 100 mg/m^2 cisplatin report peak plasma levels of about 10 M with a short half life of 20-30 min. Studies of IV infusion of 150 mg/m^2 carboplatin in humans found peak ultrafilterable Pt levels of 15 M with a half life of approximately 100-230 min.

If ultrafilterable Pt is the important species to be present during irradiation, it is then important to dose the patient 1/2 hr to 1 hr before irradiation to ensure maximal effects for this drug-radiation interaction.

Clinical Trials

The numerous experimental investigations dealing with the interaction of radiation and platinum complexes have provided the justification to initiate the use of this combination in clinical trials. It is again important to note that these trials have

been designed without knowing the precise mechanism for the interaction of Pt with radiation or the optimum timing of the radiation in relation to the platinum dosing regimen. A result of this lack of understanding in protocol design is a wide range of drug-radiation timing sequences which complicates evaluation of these early trials.

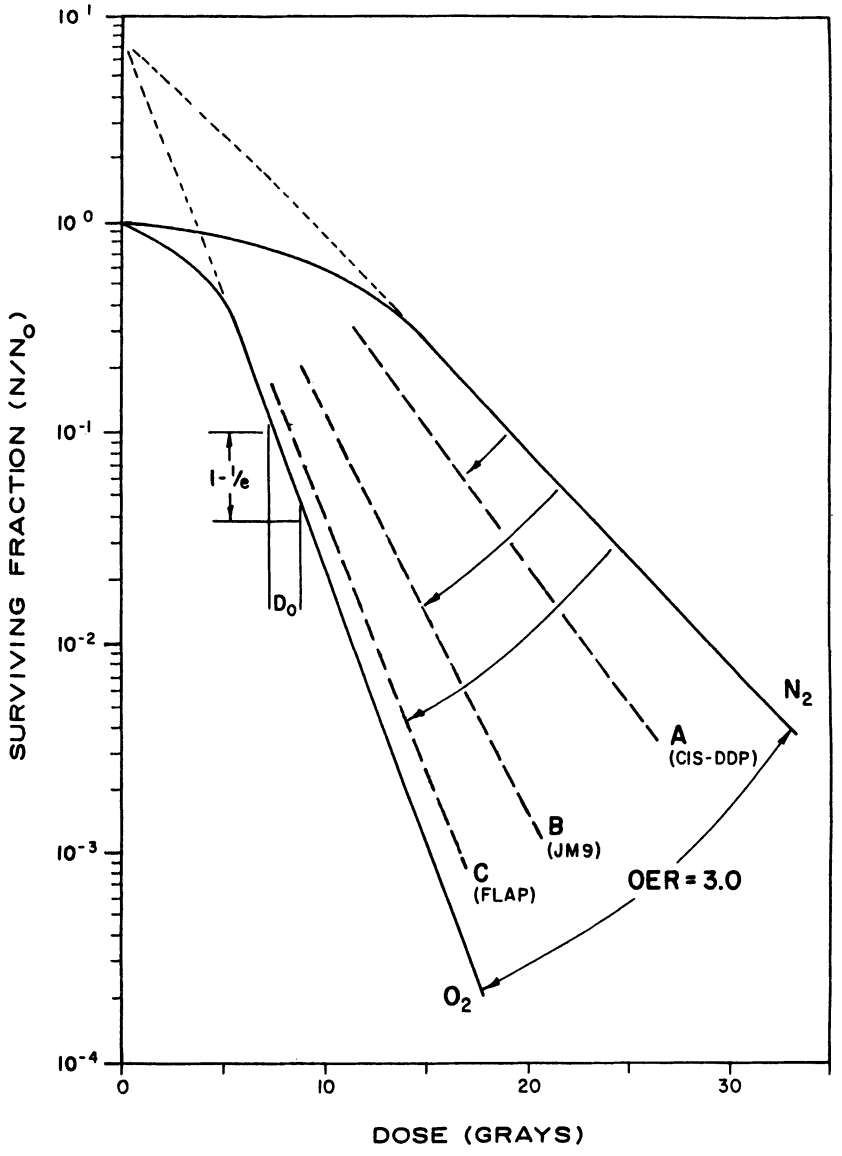
Table II outlines the published studies in 6 major tumor classes. (6) The results to date, especially in head and neck cancer and bladder cancer have provided an improvement in the quality of life and degree of local control. Whether this will translate into improved survival will be determined by the ongoing Phase III trials with cisplatin and future trials of other promising Pt and other metal drugs.

In conclusion, it is apparent that the field of inorganic chemistry has taken an additional important role in cancer therapy.

TABLE II

TUMOR	PUBLISHED STUDIES
Head and Neck	16
Bladder	6
Lung	5
Melanoma	2
Brain	3
Cervical	3

FIG. 1



RADIOSENSITIZATION OF HYPOXIC CELLS

FIG. 2

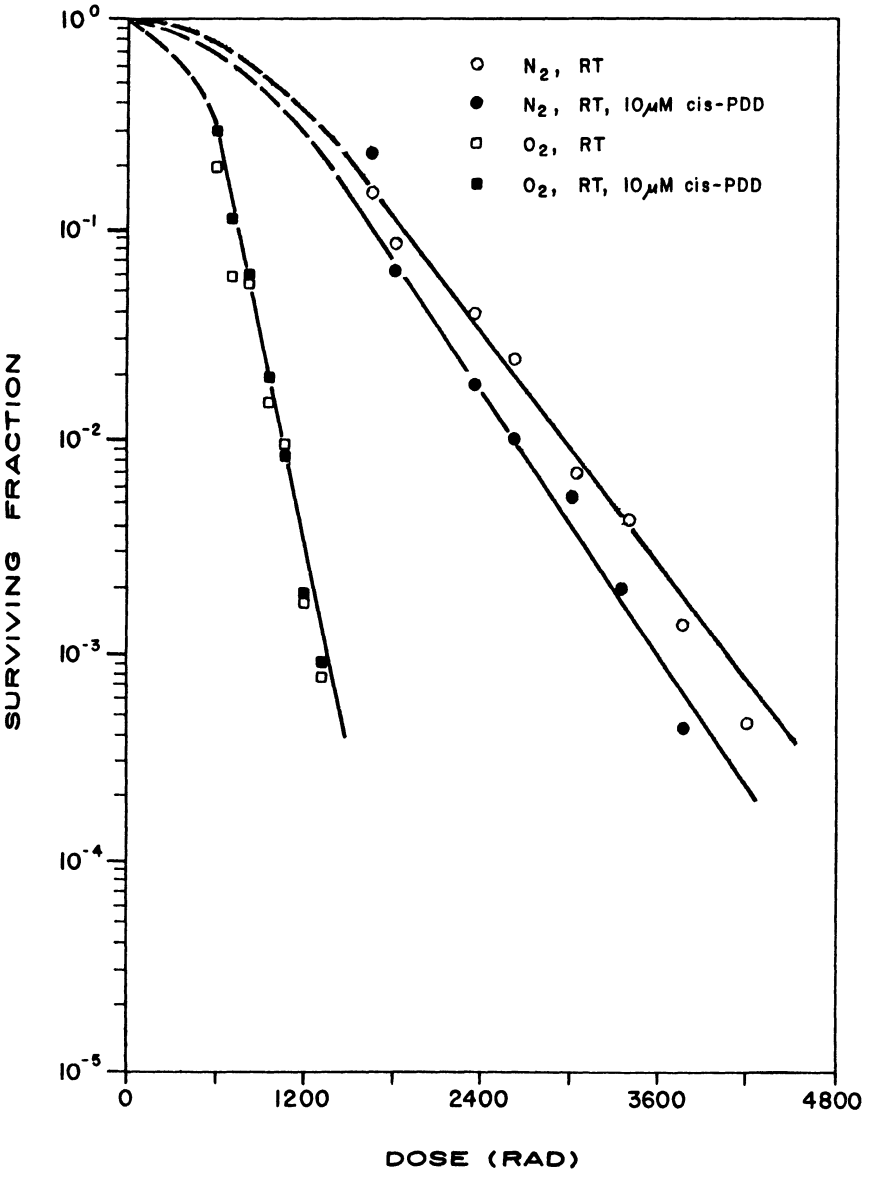
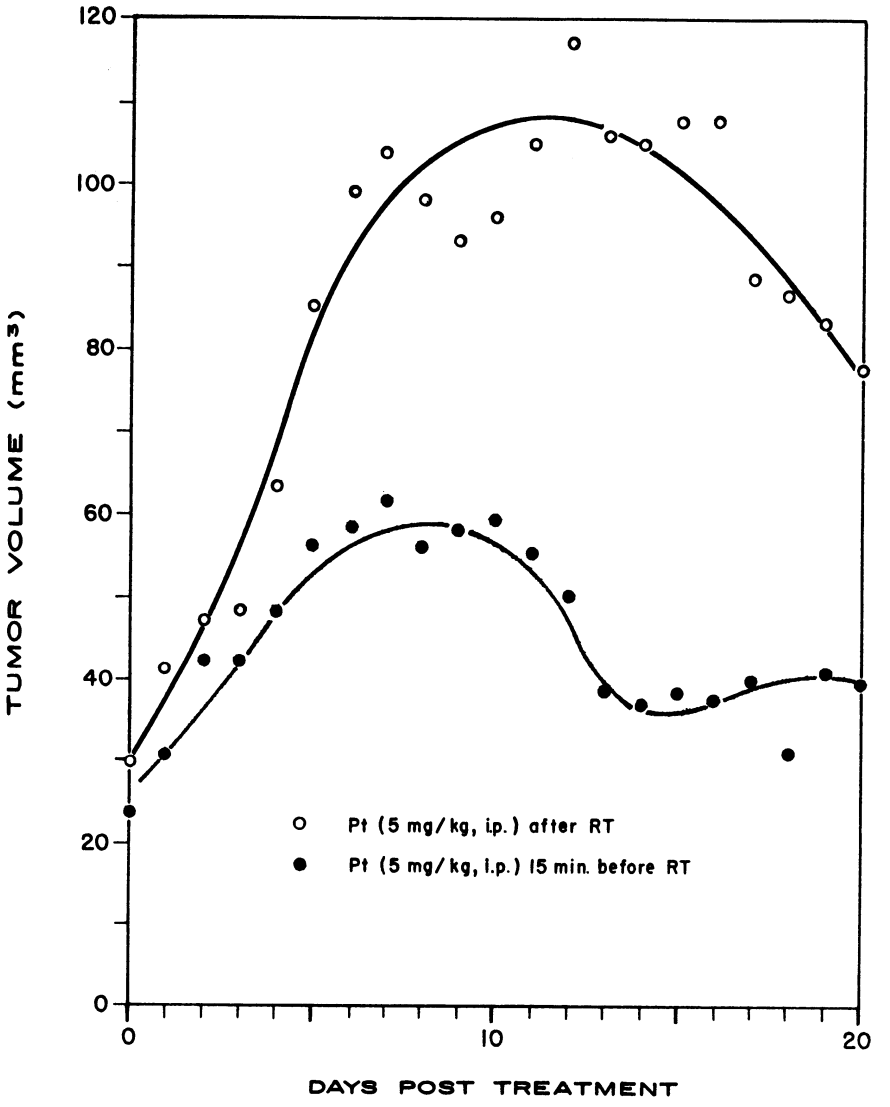
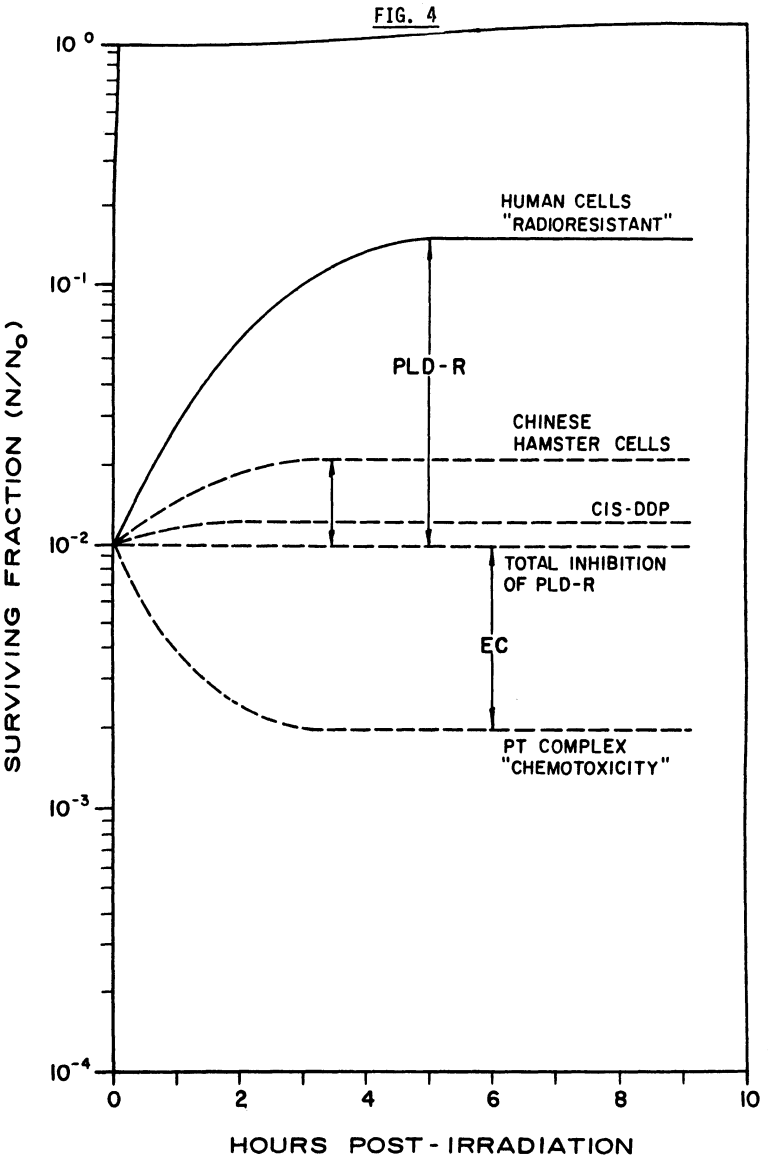

10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
SURVIVING FRACTION
0
1200
2400
3600
4800
DOSE (RAD)
N_2, RT
N_2, RT, 10μM cis-PDD
O_2, RT
O_2, RT, 10μM cis-PDD

FIG. 3


120
100
80
60
40
20
0
TUMOR VOLUME (mm³)
0
10
20
DAYS POST TREATMENT
Pt (5 mg/kg, i.p.) after RT
Pt (5 mg/kg, i.p.) 15 min. before RT



RECOVERY OF CELLS AFTER IRRADIATION

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PLATINUM COORDINATION DERIVATIVES BY THE INTRAPERITONEAL ROUTE: PHARMACOKINETICS AND CLINICAL RESULTS

W.W. Ten Bokkel Huinink, W.V.D. Vijgh, R. Dubbelman, A.P.M. Heintz, H. Franklin and J.G. McVie

Notwithstanding improvement of treatment results in ovarian cancer, even the patient who achieved a complete remission may face a relapse. The seven year follow-up figures of the study performed in the Netherlands (1), which compared cisplatin containing combination chemotherapy CHAP-5 (cyclophosphamide, hexamethylmelamine, adriamycin and cisplatin) with HexaCAF (hexamethylmelamine, cyclophosphamide, adriamycin and 5-flourouracil) show a steadily decrease of the number of disease free patients upto 60-70% of those formerly in complete remission. Therefore even for patients in complete remission as a result of cisplatin based combination chemotherapy new treatment strategies seem warranted. For the other, not reaching a complete remission this counts the more. Late intensification by an increased dose of cisplatin for instance could offer such a new treatment avenue, based on the theory of Goldie and Coldman (2). Such an increased dose might overcome acquired cisplatin resistance of remaining tumorcells. Since ovarian cancer remains limited to the abdominal space in the majority of cases and administration of drugs directly to the abdominal space offers the possibility to deliver higher dosages and higher drug exposure to the tumor site in the abdominal cavity without necessarily an increase of systemic toxicity. This cumbersome way of administrating drugs has been studied (3).

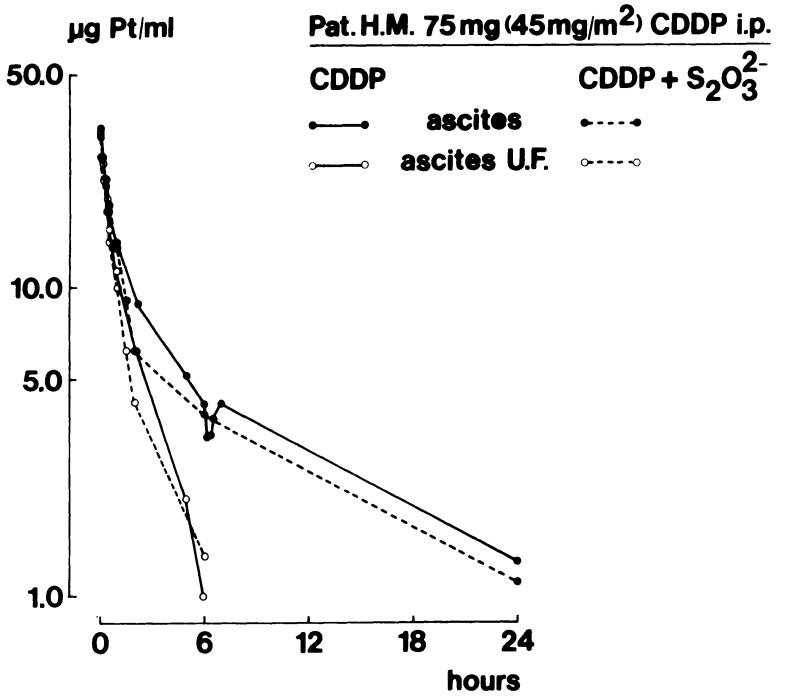
As a sequence of the study of Howell and Markman (4) we studied the antitumor effects and toxicity of intraperitoneally administered cisplatin in escalating doses with concomitant administration in-

travenously of the neutralizing agent thiosulphate when toxicity occurred. A second study involved carboplatin, which shows, even in combination treatment strategies, equal antitumor activity against ovarian cancer at a lower cost of different toxicities (5).

Cisplatin intraperitoneally and thiosulphate intravenously in minimal residual ovarian cancer

32 Patients still suffering, at restaging laparotomy, from minimal residual disease lesions of their ovarian cancer after heavy cisplatin based combination chemotherapy, were given a Tenckhoff catheter. After 7 to 10 days drug distribution into the abdominal cavity was assessed by computarized tomography of the abdomen using intraperitoneally administered contrast medium. Following this procedure the first course of cisplatin was given. With 2-4 weeks interval the patient was treated with escalating doses of cisplatin intraperitoneally, dissolved in 2 lts dialysis fluid. After 6 courses the result of this treatment was evaluated by means of a restaging peritoneoscopy or laparotomy. If toxicity occurred at a certain dose level systemic intravenous administration of thiosulphate was given concomitantly to the six hour dwell time of cisplatin given intraperitoneally. The dose of thiosulphate was a bolus of 3 g/m^2 and a constant infusion of 2 $g/m^2/hr$ during 6 hrs.
If the observed toxicity was ameliorated by thiosulphate again a dose escalation of cisplatin was tried in the next course.
Since these had been published extensively by Howell (6) we did not study the pharmacokinetics. However, as is shown in Figure I, we could not find any interference of thiosulphate with cisplatin levels measured by atomic absorbtion techniques, measuring free and total cisplatin in a couple of patients, the results of one of them are given. We could as expected demonstrate a pharmacological advantage of the i.p. administration of cisplatin.

Figure I



The importance of this procedure lies mainly in the demonstration that using such a treatment regimen, no hampering of the antitumor effect of cisplatin given i.p. was observed by the concomitant i.v. administration of thiosulphate, which in itself did not show any toxicity, since of these 32 patients 10 achieved a complete remission at restaging laparotomy. All ten had been treated with thiosulphate. Dose escalation notwithstanding severe myelosuppression or renal toxicity was possible as a result of this i.v. thiosulphate administration (7).

The dose limiting toxicities observed in this study were renal toxicity and especially invalidating neurotoxicity, inhibiting dose escalation further then 150 mg/m^2. It may be however possible to increase the dose of cisplatin further by means of the i.v. con-

comitantly administration of thiosulphate in not so heavily pretreated patients as has been shown by Howell (6). In our patients pre-existing neurotoxicity as a result of the heavy pretreatment may have played a role.
Since carboplatin lacks almost completely renal and neurotoxicity (8) a logical step after the study with cisplatin was the i.p. installation of carboplatin.

Phase I study of i.p. administrated carboplatin in ovarian cancer

Since no phase I data were available of i.p. administrated carboplatin, a phase I study had to be performed. The technical procedure of this study followed the same procedure as the study mentioned above. The patients characteristics, again only patients suffering from remaining minimal residual disease were entered are given in Table I. Again drug distribution proved to be satisfactory in all patients, notwithstanding frequent previous laparotomies and peritoneoscopies.

Table I

INTRAPERITONEAL CARBOPLATIN PHASE I

PATIENTS CHARACTERISTICS

No. patients	27
all m.r.d. ovarian cancer	
median age	54
range	27-71

no. previous CT courses (Pt)	6 (2-10)
- laparoscopies	1 (0- 3)
- laparotomies	2 (2- 5)

NKI 86.

Escalation followed a modified Fibonacci regimen as is given in Table II. Again we tried to re-evaluate the patients for antitumor effect after 6 courses. The table gives the escalation steps and number of courses in relation to the observed myelosuppression. The major toxicity encountered was myelosuppression, especially thrombocytopenia, as follows from Table II.

Table II

INTRAPERITONEAL CARBOPLATIN PHASE I

			Toxicity			
MYELOSUPPRESSION			WBC x 10^9/l.		Plts x 10^9/l.	
dose mg/m²	no. pts	no. courses	nadir	(range)	nadir	(range)
200	7	13	3.4	(2.3-5.2)	171	(69-330)
300	4	21	2.7	(2.1-5.5)	133	(20-251)
400	4	22	3.1	(1.7-9.1)	123	(39-270)
450	3	14	3.2	(2.2-4.8)	115	(31-169)
500	6	20	3.2	(0.9-5.3)	162	(6-294)
550	-	2	3.6	-	73	-
600	3	13	2.6	(1.6-4.1)	122	(26-239)

NKI 86.

Antitumor results

Only 20 patients are evaluable for response. Two complete remissions (1+, 12+ months) have been observed so far. Those patients achieving a complete remission, notwithstanding heavy previous treatment with cisplatin based combination chemotherapy with 9

courses, which resulted only in minimal residual disease, were treated at the dose level of 650 mg/m² and 700 mg/m². Taking into account the steep dose effect relation of carboplatin, no conclusions may be drawn from these treatment results.

Pharmacokinetics

The pharmacokinetics of carboplatin administered i.p. were followed meticulously and the results of this study are given in Table III.

Table III

Mean pharmacokinetic parameters (n=5) after i.p.administration of CBDCA

Parameter	Value
Dwell time (h)	4
Net dose (% D_{init})	71
$t_{\frac{1}{2}}$, UF Pt perit (h)	4.4
$Peak_{UF,pl}/Peak_{UF,per}$ (%)	5.7
$AUC_{UF,pl}/AUC_{UF,per}$ (%)	17
Perit. $Cl_{UF\ Pt}$ ($ml/min.m^2$)	3.3
$CUE_{Pt,\ 2d}$ (%D_{init})	48

As a result of this figure, the advantage of carboplatin administration i.p. seems rather small apart from the very high concentration differences and a.u.c's between ascites fluid and venous samples.

Discussion

Carboplatin seems to have advantages above cisplatin when administered i.p. since it lacks the dose limiting toxicities of cisplatin. A dose of 600-650 mg/m^2 may be recommended for further study, which is necessary to define the antitumor effect against minimal residual ovarian cancer after heavy pretreatment with cisplatin based combination chemotherapy. In order to improve the therapeutic ratio of intraperitoneal treatment with cisplatin, further studies with neutrilazing agents other than thiosulphate, which seems on the basis of this study of only limited value, however, not interfering with antitumor effect, seem warranted. Diethyldithiocarbamate is one of the candidate neutrilazing agents for such further study.

Intraperitoneal chemotherapy is still of unproven value. Further studies are needed to define its role in the treatment strategy for ovarian cancer in order to improve the now still unsatisfactory treatment results.

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CHEMOTHERAPY OF HEAD AND NECK CANCER

A. Choksi and W.K. Hong

It is estimated that in the U.S.A. in 1987, there will be 29,800 new cases of cancer of the oral cavity and pharynx and 12,100 new cases of cancer of the larynx (1). Overall 5-year survival of head and neck cancer (HNCa) is 54% (2). Patients (pts) presenting with early disease can be cured with surgery and/or XRT. These treatment methods have not provided adequate tumor control in the majority of pts who present with advanced local and regional disease. Following initial treatment, up to 60% of these pts develop local recurrences: 20% to 30% develop distant metastases, and 10% to 40% also develop second primary malignancies (3). Traditionally, chemotherapy (CT) has been used as a palliative agent in recurrent and/or metastatic tumors. Induction CT in untreated locally advanced pts is under intensive investigation at present.

CHEMOTHERAPY FOR RECURRENT OR METASTATIC TUMORS

Single Agent Chemotherapy Trials:

The most-active agents and their response rates (RR) are listed in Table 1. The relatively non-toxic regimen of weekly methotrexate (MTX) has been considered standard treatment in this group.

Table 1. Response Rates of Most Active Agents

Single Agents	Response Range %	Overall RR %
Methotrexate (MTX) (4)	8 to 63	31
Cisplatin (CDDP) (4)	14 to 41	28
Bleomycin (Bleo) (4)	6 to 45	21
Carboplatin (CBDCA) (Table 5)	14 to 26	22

Other single agents with activity in HNCa are: 5-fluorouracil(5-FU), velban(VLB), hydroxyurea(HU), cytoxan(CTX), and adriamycin(Adria).

In randomized trials, no significant difference has been shown between high-dose MTX vs low-dose MTX (5), high-dose CDDP vs low-dose CDDP (6), and MTX vs CDDP (7).

Combination Chemotherapy Trials:

Different combinations have been tried in recurrent HNCa: Non-CDDP containing regimens (Table 2) and CDDP combination regimens (Table 3). Although RRs have been increased with CDDP-containing regimens, no randomized trial has shown superiority of combinations over single agents in achieving overall survival benefits (Table 4).

Table 2. Non-CDDP Containing Regimens

Combinations	Response Range %	Overall RR %
CTX+Bleo+MTX+5FU (8-12)	11 to 69	45.0
MTM+VCR+Bleo (13,14)	25 to 54	36.8
Price Hill regimen (15,16)	13 to 41	35.5
MTX+5FU (17-21)	16 to 65	-

MTM-C = Mitomycin-C

Table 3. CDDP-Containing Regimens

Combinations	Response Range %	Overall RR %
CDDP+Bleo (22,23)	13 to 55	25.7
CDDP+Adria+CTX (46,47)	7 to 64	43.5
CDDP+VCR+Bleo (24,25)	30 to 50	38.1
CDDP+Bleo+MTX (26-31)	27 to 100	58.8
CDDP+VLB+Bleo (32,33)	45 to 63	50.0
CDDP+5FU (34-45)	11 to 75	48.5

In a British randomized trial with 4 arms: 1) No treatment, 2) Bleo, 3) CDDP, 4) CDDP + Bleo: CDDP-alone and CDDP + Bleo arms did signficantly better in terms of time at home and overall survival (53).

Platinum Analogues:

CDDP is one of the most active CT agents in HNCa. Although a randomized trial of 60 mg/m2 to 120 mg/m2 CDDP did not show any differ-

ence, there is some evidence that CDDP at high doses (200 mg/m2) may produce high RRs (54,55), but toxicity (neurotoxicity, nephrotoxicity, myelosuppression) at that dosage becomes quite severe. Newer analogues with minimal neuro- or nephrotoxicity may prove of immense value in HNCa. In Phase II trials with CBDCA alone, RRs have varied from 14% to 26% (Table 5). In combination trials, CBDCA + 5FU has yielded responses of 27% to 48% (60,61) and CBDCA + CDDP has yielded a 40% RR (62). The results are quite encouraging and further trials with CBDCA are anticipated.

On the other hand, Iproplatin in Phase II studies have yielded low RRs, but further studies are awaited (Table 7) (53-54).

Table 4. Combination vs Single Agent Chemotherapy Response and Survival and Rates

Authors	Drugs	No. Pts	RR %	Median Survival (months)
DeConti, et al (48)	MTX vs	81	26	5.0
	MTX+LV vs	80	24	4.4
	MTX+LV+CTX+AraC	76	18	3.3
Jacobs, et al (49)	CDDP vs	41	18	6.2
	CDDP+MTX	39	33	6.9
Drelichmen, et al (50)	MTX vs	24	33	5.6
	CDDP+VCR+Bleo	27	41	4.0
Vogl, et al (51)	MTX vs	83	35	5.6
	CDDP+Bleo+MTX	80	48	5.6
Williams, et al (52)	MTX vs	98	16	7.3
	CDDP+VLB+Bleo	92	24	6.8

Table 5. Single Agent Carboplatin Trials in Recurrent or Metastatic Head and Neck Cancer

Investigator	Pt No.	CR	PR	Overall Response(%)
Eisenberger (56)	31	2	6	8 (26.0)
Creekmore (57)	14	0	2	2 (14.0)
Ohnuma (58)	13	0	2	2 (15.0)
Al-Sarraf (59)	29	3	4	7 (24.0)
Total	87	5	14	19 (22.0)

Table 6. Carboplatin Combination Trials in Recurrent or Metastatic Head and Neck Cancer

Investigator	Combinations	Pt No.	CR + PR	Response Rate CR+PR %
Forastiere (51)	CBDCA + 5FU	27	3 + 10	48.0
Oliver (52)	CBDCA + 5FU	26	2 + 5	26.9
Trump (53)	CBDCA + CDDP	5	0 + 2	40.0

Table 7. Iproplatin Trials in Recurrent or Metastatic Head and Neck Cancer

Investigator	Pt No.	Major Response	Response Rate CR+PR %
Abele (EORTC) (54)	39	0	0
Al-Sarraf (55)	34	4	12

Although the response rates are approaching 50% with the use of newer combination regimens, CRs have only been 5% to 15% with short response durations. Thus, in future trials it is important to show that, in spite of its toxicity, chemotherapy improves survival and/or the quality of life.

Future Directions For Recurrent Tumors:

1. Phase II trials of new agents for more effective CT.
2. Phase II trials of new CT combinations.

CT combinations which need to be studied further are: 1) CBDCA + 5FU, 2) CBDCA + CDDP, 3) CBDCA + Bleo, and 4) Non cross-resistant regimens, e.g., CDDP + 5FU alternately with CBDCA + Bleo.

INDUCTION CHEMOTHERAPY IN LOCALLY ADVANCED HEAD AND NECK CANCER:

CT after recurrence gives poor RRs; however, clinical trials of previously untreated pts indicate higher RRs.

Theoretical advantages of induction chemotherapy are: 1) increased cell kill due to intact vascularity, 2) increased tolerance due to better performance status and nutritional status, 3) potential eradication of sub-clinical metastatic disease, and 4) efficacy of local treatment enhanced by cytoreduction.

Single Agent Trials:

Single agent induction CT trials were started as early as 1960's. Since then, a number of trials have been done with MTX as single agent; however, randomized trials have not shown any survival benefit. One non-randomized study showed a RR of 52% and demonstrated increased resectability and increased disease-free status in responders (65). Bleomycin (Bleo) and CDDP were also tried as single agent induction CT; however, complete response (CR) rates with single agents remained low ($\leq$5%).

Combination Chemotherapy Trials:

Most induction trials have used CDDP-containing regimens; however, **Price and Hill** used a non-CDDP containing regimen of MTX + VCR + Bleo + 5FU + Hydrocortisone and achieved a 66% RR; CR following local therapy was significantly greater (78%) and median survival of CT responders was significantly better than nonresponders (66).

In the late 1970's, a CDDP combination, first used by **Randolph et al** as induction CT, obtained a 71% RR and 20% CR (69). Since then, a number of trials using different CDDP combinations have been reported. CDDP + 5FU appears to be a more effective and less toxic regimen than other regimens reported. Overall RRs are reported in Table 8.

Table 8. Response Rates of Combination Cisplatin Regimens

	Pts	CR %	PR %	Overall RR %
CDDP (67,68)	40	4	36	40
CDDP+Bleo (69-73)	447	13	50	63
CDDP+Bleo+MTX (74-78)	199	14	64	78
CDDP+Bleo+VCRorVLB (79-83)	282	21	52	73
CDDP+5FU (84-91)	291	35	52	87

Importance of Achieving a CR:

Several studies have shown that CRs have much better survival than partial responders or nonresponders. Whether attainment of CR just identifies a subset of pts who have favorable outcome regardless of treatment remains controversial.

It has been shown that approximately 50% of pts with

clinical CR are pathologic CR, thus indicative of the need for more local therapy with surgery and/or XRT. A recent study has suggested that pts with pathologic CR may avoid radical surgery and be treated only with XRT (87).

Sequential Chemoradiotherapy:

Use of CT and XRT has allowed us to monitor and correlate the sequential response patterns produced by each modality of treatment. It has been shown that CT responders have further response to XRT and CT nonresponders predict poor response to XRT (92). In fact, non-randomized studies have shown high local control rates with sequential CT and XRT approach (66,69,71,93). However, 4 randomized trials published so far have not shown any survival benefit (Table 9). Three of them used only single agent CT; the fourth trial used the **Price and Hill** regimen which has been reported to have low CRs (<10%). A randomized study with an effective combination regimen is needed to establish the role of the sequential CT + XRT approach.

Simultaneous Chemoradiotherapy:

Chemotherapy and radiotherapy have been tried simultaneously to evaluate the potential of CT to function as a radio-sensitizing agent. Randomized trials have failed to show benefit for simultaneous CT + XRT over XRT alone (98-101); however, a preliminary report of a randomized study of MTM-C + XRT vs XRT alone suggests improved disease-free survival benefit in the MTM-C arm (102). Also several non-randomized trials of CDDP $\pm$ 5FU + XRT show high RRs (103-106). This approach definitely seems warranted in inoperable HNCa.

RANDOMIZED TRIALS OF INDUCTION CHEMOTHERAPY

Induction CT + Local Therapy (Surgery and/or XRT) vs Local Only:

So far, there have been six randomized trials published (Table 10) (73,90,107-110). Although none have shown survival benefits, the following points need to be taken into consideration when evaluating the trials: 1) Use of single or ineffective induction regimens in most studies, 2) In studies where fairly effective regimens were used, low optimal doses or inconsistent schedules have resulted in very low CRs, 3) Possible attenuation of XRT and/or surgical treatment in CT group, 4) Inadequate follow-up.

Table 9. Sequential CT + XRT vs XRT-Alone Therapy

Investigator	Regimens	Pt No.	Survival Benefit
Von Essen 1968 (94)	MTX + XRT vs 5FU + XRT vs IUDR + XRT vs XRT alone	87	None
Knowlton 1975 (95)	MTX + XRT vs XRT alone	96	None
Fazekas 1980 (96)	MTX + XRT vs XRT alone	638	None
Stell 1983 (97)	Price Hill regimen + XRT vs XRT alone	86	None

Table 10. Randomized Trials of Induction Chemotherapy

Investigator	Regimen	Pt No.	CR + PR	Survival Benefit
Holoye 1984	Bleo+CTX+MTX+5FU	83	4 + 62	None
Schuller 1984	CDDP+Bleo+VCR	146	20 + 25	None
Taylor 1985	MTX	90	6 + 34	None
Haas 1985	CDDP+5FU	59	19 + 70	None
Martin 1986	CDDP+Bleo+MTX+5FU	60	7 + 57	None
Wolf 1987	CDDP+Bleo	411	8 + 40	None

Obviously, further controlled studies with effective regimens, adequate sample sizes, and adequate follow-up are necessary.

FINDINGS FROM INDUCTION CHEMOTHERAPY TRIALS

Positive points which have been shown in induction CT trials:

1. Does not increase surgical complications or compromise XRT.
2. Response to CT correlates with tumor burden.
3. Response to CT predicts further response to XRT.
4. High CR rate is obtainable with effective regimens.
5. Pathologic CR in 25% to 70% of clinical CR patients.
6. Radical surgery may be omitted in CR.

Negative points which have been shown in induction CT trials:

1. CT prolongs treatment course.
2. Treatment is expensive.
3. Surgical margins are difficult to assess after major response.
4. High rate of patient refusal to undergo local therapy.
5. No improvement in survival in randomized trials, yet.
6. Later palliative CT may be compromised.

ONGOING TRIALS IN U.S.A.

Several clinical trials of adjuvant treatment in HNCa are currently ongoing:

Head and Neck Intergroup Study:

This is a randomized trial investigating the value of postoperative adjuvant CT in surgery and XRT in operable stage III or IV HNCa. The CT regimen is CDDP 100 mg/m2 intravenously and 5FU 1000 mg/m2 per day continuous infusion for 5 days every 3 weeks for 3 courses. Adjuvant CT has been given both following surgery and prior to XRT.

VA Cooperative Laryngeal Cancer Study:

CT has other potential roles that deserve exploration in addition to improving overall survival. Those pts who fortunately survive often face a lifetime of significant morbidity from the cosmetic and functional debilities of surgical resection. If the use of induction CT could modify or eliminate surgical resection, the quality of life of these pts might improve, even if the survival rate was only equivalent.

This question is currently being addressed by the VA Cooperative Laryngeal Cancer Study in a randomized clinical trial to determine whether induction CT followed by XRT is an alternative to or an improvement upon laryngectomy and postoperative XRT. The experimental group will receive induction CT with CDDP + 5FU for a minimum of 2 cycles, after which tumor response will be assessed. Pts showing no response will receive surgery followed by XRT. Pts showing response will receive 1 more cycle of CT and then XRT. Pts showing persistent disease after XRT will have salvage laryngectomy. The objective of this study is to investigate the value of sequential CT and XRT in laryngeal cancer.

Chemoprevention Trial with Retinoids

This study is investigating the value of 13-cis retinoic acid as a cytostatic agent after surgery and/or XRT in pts who are at risk for local recurrence and second primary neoplasms. This study is ongoing at M.D. Anderson Hospital as a double-blind randomized trial. The dose of 13-cis retinoic acid is 50-100 mg/m2 orally, with a treatment period of 12 months.

Future Directions For Induction Chemotherapy:

1. Development of effective regimens to increase CR rates, e.g., non cross-resistant CDDP and CBDCA combinations.
2. Early and/or late intensification to decrease relapse rates.
3. Sequential CT + XRT in nasopharyngeal carcinoma.
4. Use of intra-arterial treatment as induction therapy.
5. Interdigitation of CT and XRT.
6. Post-operative adjuvant CT trials.
7. Value of additional CT in responders.
8. Combined use of cytotoxic and cytostatic agents.

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DIFFERENT PLATINUM SCHEDULES IN THE TREATMENT OF EPITHELIAL OVARIAN CANCER

C. Mangioni, N. Colombo, A. Epis, F. Landoni, L. Redaelli, L. Vassena and W. Torri

INTRODUCTION.

Cisplatin is the most active single agent in the treatment of epithelial ovarian cancer.

Since the first report of its activity (1), several efforts have been made to try to identify the most effective schedule and to combine cisplatin in multiple drug regimens with hoped increased efficacy. No clear evidence exists that combination therapy is more effective than single agent cisplatin in producing long term survival in patients with advanced epithelial ovarian cancer.

Experimental and clinical data suggest a steep dose-response relationship for cisplatin in human ovarian cancer (2,3). Responses could be achieved in patients resistant to conventional doses by increasing the dosage to 100-200 mg/mq. (4). Unfortunately, nephrotoxicity, neurotoxicity and otoxicity are important limiting factors in reaching higher and more effective doses of cisplatin.The concept of dose-intensity has been recently introduced and seems to correlate with median survival times and tumor response rates.

Dose intensity (=dosage of a therapeutic agent administered per unit time) may be increased by shortening the interval between courses, keeping the dosage low and thus avoiding undue toxicity.

The aim of this paper is to review our previous experience on the use of single agent cisplatin in patients with epithelial ovarian cancer.

As a result of the developing concept of dose-intensity, different doses and schedules have been sequentially used as part of controlled clinical trials.

The historical comparison of response-rate and toxicity of three different regimens will be presented.

MATHERIAL & METHODS.

Since 1980 a monochemotherapy with cisplatin (P) has been administered to patients with ovarian cancer as one of the regimens of controlled clinical trials carried out in our Institution. Three protocols included a total of 181 patients treated with cisplatin alone at different schedules and doses: in the first study cisplatin was given at 50 mg/mq i.v. every 28 days for 6 cycles (P50). This regimen was compared with cisplatin + cyclophosphamide and with cisplatin + cyclophosphamide + adriamycin. In the second study cisplatin 100 mg/mq i.v. every 28 days for 6 courses was compared with carboplatin. Finally a non randomized clinical trial tested the efficacy of an induction therapy with cisplatin 1 mg/Kg weekly for 6 weeks (PW) followed by 3-4 cycles of cisplatin + cyclophosphamide 600 mg/mq i.v. + esorubicin 50 mg/mq i.v. every 28 days (PEC). The following table shows the main characteristics of patients in the 3 regimens:

Table 1.

PATIENT CHARACTERISTICS

	P50 PTS (%) (91)	P100 PTS (%) (31)	PW PTS (%) (59)
HISTOTYPE			
SEROUS	62 (68.2)	17 (54.9)	41 (69.5)
MUCINOUS-			
-ENDOMETRIOID	14 (15.3)	8 (25.8)	8 (13.6)
INDIFFERENT.	10 (11)	5 (16.1)	9 (15.2)
CLEAR CELLS	5 (5.5)	1 (3.2)	1 (1.7)
FIGO GRADE			
I	16 (17.6)	-	4 (6.8)
II	27 (29.7)	9 (29)	7 (11.9)
III	48 (52.7)	22 (71)	48 (81.3)
STAGE III	73 (80.2)	27 (87.1)	48 (81.4)
IV	18 (19.8)	4 (129	11 (18.6)
RESIDUAL TUMOR			
2	24 (26.4)	6 (19.4)	11 (12.6)
2	67 (73.6)	25 (80.6)	48 (81.4)
AGE			
45	12 (13.2)	3 (09.7)	5 (08.4)
45-65	60 (66.0)	22 (71)	27 (45.8)
65	19 (20.8)	6 (19.3)	27 (45.8)

While responding patients in the first protocol (P50) were evaluated with surgical procedures after 6 courses of treatment, an interval evaluation of response was done after 6 weeks of PW and after 3 courses of P100. Patients with no clinical evidence of disease were proposed for an early surgical evaluation 60 days (PW) and 80 days (P100) respectively from the beginning of therapy. All responding patients were moreover surgically evaluated at the end of the therapeutic program.

RESULTS.

The early results of therapy in patients treated with the 2 regimens PW and P100 are shown in the following table:

Table 2.

	PW*	P100**
	PTS (%)	PTS (%)
C R	**11** (18.6)	**8** (28.6)
CLINICAL	3	3
PATHOLOGIC	8	5
P R	**40** (67.8)	**14** (50)
CLINICAL	21	6
PATHOLOGIC	19	8
NO RESPONSE	**8** (13.56)	**6** (19.35)
CLINICAL	7	4
SURGICAL	1	2
NOT EVALUABLE	-	**3**
TOTAL	**59**	**31**

* PW Evaluation at 60 days from the beginning of therapy (total DDP 210 mg/mq in 6 weeks cycles)

**P100 Evaluation at 90 days from the beginning of therapy (total DDP 300 mg/mq in 3 monthly cycles).

Even though not all complete responses were confirmed by surgery, it is noteworthy the high overall response rate (PW=86.4%, P100=71%) after such a short period of treatment.

The final results of the three therapeutic programs are the following:

Table 3.

	FINAL RESPONSE		
	P50	**P100**	**PW+PEC**
	PTS (%)	PTS (%)	PTS (%)
C R	**17** (18.7)	**6** (19.4)	**17** (28.8)
PATHOLOGIC	16	6	17
CLINICAL	1	0	0
P R	**30** (33)	**16** (51.6)	**30** (50.9)
PATHOLOGIC	29	16	15
CLINICAL	1	0	15
NO RESPONSE	**44** (48.3)	**9** (29.0)	**12** (20.3)
TOTAL	**91**	**31**	**59**

The PEC polichemotherapy regimen after PW was able to further improve 18 partial responses and to convert 7 partial responses in complete. A similar multiple drug regimen was also used in most patients with persistent disease after 6 courses of P50 and P100. The following table shows the contribute of second line therapy in achieving complete responses after single agent cisplatin:

Table 4.

		C R DDP alone	**C R** DDP+PAC/PEC
		PTS (%)	PTS (%)
P50	(91 PTS)	17 (18.7)	19 (20.0)
P100	(31 PTS)	6 (19.4)	8 (25.8)
PW	(59 PTS)	11 (18.6)	17 (28.8)

No patients progressing on single agent cisplatin responded to polichemotherapy.

All these regimens were well tolerated. In particularly PW was safely administered even to old patients with poor performance status.

Table 5.

SINGLE AGENT CISPLATIN IN DIFFERENT SCHEDULE

HEMATOLOGIC TOXICITY

	P50 PTS (%)	P100 PTS (%)	PW PTS (%)
NEUTROPENIA			
WHO G1	26 (28.6)	8 (25.8)	28 (47.4)
G2	7 (7.7)	6 (19.3)	7 (11.8)
G3	1 (1)	1 (3.2)	3 (5)
			p = 0.02
THROMBOCYTOPENIA			
WHO G1	8 (8.8)	2 (6.4)	8 (13.5)
G2	1 (1)	1 (3.2)	1 (1.7)
ANEMIA			
WHO G1	27 (29.7)	13 (41.9)	22 (37.2)
G2	4 (4.4)	6 (19.3)	17 (28.8)
G3	1 (1)	1 (3.2)	-
			p = 0.0002

Neutropenia and anemia were more severe in patients receiving P100 and PW than in patients treated with P50, while thrombocytopenia was comparable in the three regimens. No other major side effects were observed. Nausea and vomiting were generally controlled by antiemetic agents in the two most recent protocols (PW & P100). No severe neurotoxicity nor neprhotoxicity was observed.

Table 6.

OTHER TOXICITIES

	P50 PTS (%)	P100 PTS (%)	PW PTS (%)
NAUSEA & VOMITING			
WHO G1	12 (19.2)	1 (3.2)	2 (3.4)
G2	34 (37.3)	29 (93.5)	46 (77.9)
G3	25 (27.5)	0	1 (1.7)
CREATININE			
WHO G1	8 (8.8)	3 (9.7)	7 (11.8)
G2	1 (1)	-	4 (6.7)
PERIPHERAL NEUROPATHY			
WHO G1	5 (5.5)	1 (3.2)	1 (1.7)

FINAL REMARKS.

The above exposed data confirm the efficacy of single agent cisplatin given in a monthly schedule to patients with epithelial ovarian cancer (5). The overall response rate of 86% (clinical + surgical) observed in patients treated with weekly cisplatin indicates that this regimen is an active induction therapy. The early evaluation of response (after 6 weeks of PW and 3 courses of P100) highlights that responses to cisplatin are usually precocious and that the overall response rate is not improved by continuing the same regimen for a longer period of time. Further chemotherapy with a multidrug regimen can improve already achieved responses but seldom is effective in resistant tumours.

There is evidence from clinical data on both advanced breast cancer patients and advanced ovarian carcinoma patients that suggests that higher chemotherapy dose intensity (increased dose per unit time) might beneficially affect treatment outcome (6). In our experience weekly cisplatin has been a monochemotherapy schedule of easy administration and good tolerance even for old patients with poor performance status. It produced response rates similar to those achieved with P50 and P100 in a shorter period of time. Nevertheless, the scanty results of subsequent second line therapy highlight the problem of an early development of drug resistance. The mild toxicity encountered encourages further investigation of a weekly induction therapy with cisplatin in combination with other drugs to timely prevent the early development of drug resistance with more aggressive regimens.

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CHEMOTHERAPY OF LUNG CANCER

A. Paccagnella, G. Favaretto and M.V. Fiorentino

Lung cancer is the leading cause of cancer death among men; in women it is going to surpass breast cancer and to become the primary cause of cancer death. The 5 year survival of patients with lung cancer ranges from 5% to 10%. One of the reason for this high mortality is that early detection is difficult and the majority of patients present with unresectable disease. Surgery and radiotherapy have probably achieved the best of their present possibilities and advances are mainly expected from chemotherapy. Small Cell Carcinoma (SCLC), differs both biologically and clinically from Non Small Cell Carcinoma (NSCLC).

While SCLC is highly responsive to chemotherapy with a small but definite cure rate the role of chemotherapy in NSCLC remains unclear. Besides histologic difference, factors influencing therapeutic results are represented by staging (patients with limited disease responding generally better than patients with extensive disease) and by other patient characteristics like performance status weight loss, prior treatment and time from diagnosis to therapy.

SMALL CELL LUNG CANCER

In Small Cell Lung Cancer, a series of drug combinations can produce response rates ranging from 60% to 90% with complete

responses between 20% and 40% depending on the disease stage. Cohorts with disease limited to the primary site and to the regional lymph nodes can reach up to 20% of long survivors with median survival superior to one year. In patients with disseminated disease, chemotherapy can induce short duration responses only.
The traditional most active single agents in SCLC are listed in Table 1.

TABLE 1 - TRADITIONAL SINGLE AGENTS IN SCLC

drug	No. of pts	responders
HN_2	55	44%
CYCLOPHOSPHAMIDE	189	22%
DOXORUBICIN	36	31%
VINCRISTINE	43	42%
METHOTREXATE	73	30%
CCNU	76	14%

Although frequently employed as first line treatment in phase II studies they rarely produced complete responses. (1, 2).
Table 2 shows the results of new active agents in untreated and pretreated patients. It is still unclear whether they are no-cross resistant, more active or less toxic in comparison with the parent compounds (Iphosphamide vs Cyclophosphamide, Vindesine vs Vincristine, ACNU vs CCNU and Etoposide vs Teniposide)(3, 4).

TABLE 2 - NEW ACTIVE AGENTS IN SCLC (PLATINUM DERIVATES EXCLUDED)

drug	Untreated pts	resp.	Treated pts	resp.	Overall pts	resp
IPHOSPHAMIDE	17	47%	9	44%	26	46%
EXAMETHYLMELAMINE	26	42%	19	37%	45	40%
VINDESINE	--	--	74	19%	74	19%
ETOPOSIDE (VP16)	167	45%	95	12%	262	33%
TENIPOSIDE (VM 26)	26	54%	79	14%	105	24%
ACNU	22	59%	20	15%	42	38%

Cis Platin (CDDP) as single agent has received extensive evaluation with different dose schedules (75-140 mg/m^2 q 3-4 weeks). A total of eight trials with 139 treated patients have been reported. All but 4 patients had received prior chemotherapy. The cumulative response rate of 15% with 2 complete responses indicates that CDDP is an active agent. It is noteworthy that 3 out 4 previously untreated patients responded to CDDP (5, 6, 7, 8, 9, 10, 11, 12).

The second generation platinum analogues, Carboplatin (JM-8) and Iproplatin (JM-9), are characterized by a different pattern of drug toxicity. The dose limiting side effects are myelosuppression, vomiting and diarrhea. Nephrotoxicity, neurotoxicity and ototoxicity are much less severe than with equivalent doses of CDDP, and seem non-additive.

Carboplatin has been used in the treatment of 73 patients with advanced disease at doses between 300 and 400 mg/m^2 (13, 14, 15). The cumulative response rate in pretreated patients (20%) is similar to that obtained with cisplatin. In untreated patients however, the response rate was 57%, with 3 (6%) complete responses (Table 3).

TABLE 3 - SECOND GENERATION PLATINUM COMPOUNDS IN SCLC

	UNTREATED		TREATED		OVERALL	
	PTS	RESP.	PTS	RESP.	PTS	RESP
CARBOPLATIN						
SMITH, 1985 300-400 mg/m^2 q 4 weeks (13)	30	18(60%)	26	5(19%)	56	23(41%)
WINKLER, 1985 320-400 mg/m^2 q 4 weeks (14)	6	-(0%)	-	-	6	-(0%)
JACOBS 1986 400 mg/m^2 q 4 wks (15)	11	9(82%)	-	-	11	9(82%)
GORE, 1986 800-1200-1600 mg/m^2 (16)	5	5	-	-	5	5
IPROPLATIN						
MADAIEWICZ, 1984 270 mg/m^2 q 3-4 weeks (17)	2	2(100%)	5	2(40%)	7	4(57%)

In a high dose phase I study escalating Carboplatin from 800 to 1600 mg/m^2, Gore obtained 2 complete and 3 partial responses in 5 untreated patients. Renal toxicity was mild and reversible. Peripheral neuropathy and ototoxicity did not occur. Myelosuppression was the major toxicity in all patients (13, 14, 15, 16).

Iproplatin has been less extensively tested in SCLC. Preliminary data however, show activty and toxicity patterns similar to those of Carboplatin (17).

In vitro studies and clinical trials have suggested a dose-response relationship for CDDP in solid tumors, as ovarian and testicular cancer resistant to standard dose CDDP (18, 19). The dose

limiting factors are represented by renal and neuro-toxicity. Attempts to decrease the toxicity with dose schedules and hydration regimen modifications seem promising (20; 21).

Little is known about the efficacy of high doses of CDDP in SCLC. Recently Von Hoff, using a human tumor cloning technique, constructed in vitro dose response lines for 4 drugs including CDDP, against SCLC colonies. Cisplatin produced the greatest slope suggesting a strong dose-response relationship (22).

Since Carboplatin and Iproplatin are lacking of renal and neuro toxicity, it would be of considerable interest to test high doses of these Platinum analogues in a drug intensification program with or without autologus bone marrow trasplantation.

The Doxorubicin, Cyclophosphamide and Vincristine (CAV) combination as first line regimen in SCLC has been the most used regimen in the Seventies.

In limited disease it produced a response rate of approximatively 60-80% and a median survival of 12-18 months with 10-15% long term survivors depending on dose delivered, number of administered cycles, chemotherapy at relapse and chest irradiation. In extensive disease the response rates ranged between 40% and 60% with a median survival of 6-10 months and less than 5% long term survivors.

To improve results and reduce toxicity, a series of studies have been done modifying the CAV regimen with the inclusion of Etoposide, one of the most active single agents in phase II studies.

The standard CAV regimen has been tested in randomized studies against similar regimens with the substitution of Etoposide for Vincristine (CAV vs CAE) and for Doxorubicin (CAV vs CEV) or with the addition of Etoposide (CAV vs CAVE) (23, 24, 25, 26).

In these trials, combinations including Etoposide produced a small but reproducible advantage in terms of response rate, time to

progression, response duration and median survival, particularly in the extensive stage subgroup.

Cisplatin plus Etoposide (CDDP + VP16) is a highly sinergistic combination in many animal and human models including Small and Non Small Cell Lung Cancer. As second line treatment after CAV, in resistant or relapsing patients, CDDP + VP16 is the only combination giving a reproducible 50% objective response rate (Table 4) (27, 28, 29, 30, 31, 32, 33).

TABLE 4 - CDDP + VP16 FOR RESISTANT/RELAPSING SCLC

		prior CT	pts	resp.
SIEROCKY, 1979	(27)	CAV	6	83%
LOPEZ, 1982	(28)	multiple drugs	8	63%
EINHORN, 1984	(29)	CAV	32	81%
BATIST, 1984	(30)	multiple drugs	18	11%
EVANS, 1985	(31)	CAV	78	55%
PORTER, 1985	(32)	CAV	26	52%
FIGOLI, 1985	(33)	multiple drugs	25	46%

Studies using this combination with different dosage schedules as first line therapy seem to produce overall and complete response rates superior to those obtainable with CAV, and comparable response duration and survival (Table 5) (34, 35, 36, 37, 38, 39, 40, 41).

TABLE 5 - CDDP + VP16 AS 1st LINE TREATMENT IN SCLC

		pts	response rate complete	overall
SIEROCKY, 1979	(34)	38	47%	94%
KIM, 1982	(35)	24	25%	88%
WILKE, 1985	(36)	24	75%	96%
BONI, 1985	(37)	22	59%	95%
SCHER, 1985	(38)	37	54%	84%
EVANS, 1985	(39)	28	43%	86%
SALVATI, 1985	(40)	18	39%	89%
IHDE, 1987	(41)	41	22%	80%

The superiority of CDDP + VP16 over CAV remains, however, to be demonstrated with randomized studies. It is of interest to note that CAV used after DDP + VP16 front line treatment, seems non effective.

Scher observed no objective responses to CAV in 18 patients pretreated with CDDP + VP16, Evans observed 3 out 12 (25%) and Sculier 3 out 37 (8%) objective responses only (38, 42, 43).

These data question the assumed non-cross-resistance between the CAV and CDDP + VP16 combinations and introduce the new concept of "monodirectional" non-cross-resistance.

Based on the Goldie and Coldman mathematical model of drug resistance a series of studies have been done to verify the assumption that the early use of alternating non-cross-resistant drug combinations would improve therapeutic results.

Studies using alternating regimens not fulfilling the criteria of non-cross-resistance, failed to show any advantage in response rate and survival in comparison with standard regimens (45). Based on the assumption that CAV and CDDP + VP16 regimens seemed to be equally active and non-cross-resistant the were exploited in a series of studies testing the Goldie and Coldman hypothesis (Table

6). It is difficult, however, to compare the different studies because they differ in number of delivered cycles, drug dosage schedule, intervals, and type of maintenance therapy.

Preliminary data from 3 multicentric randomized studies are now available.

A study from NCI-C compared alternating versus sequential chemotherapy (3 courses of CAV followed by 3 courses of CDDP + VP16) in limited disease patients. No difference was found in response rate and survival between the two regimens (45).

The same group in a trial comparing CAV as standard treatment versus the alternating CAV and CDDP + VP16 in extensive disease patients found a significantly superior response rate and progression free survival, and a marginally superior overall survival in favour of the alternating regimen (49).

A third randomized study by SWOG comparing alternating chemotherapy against concurrent chemotherapy (ADR 40 mg/m^2, VCR 1,4 mg/m^2, CTX 750 mg/m^2 day 1 and VP16 75 mg/m^2 days 1; 2 and 3) in limited disease patients failed to show any advantage for the alternating chemotherapy regimen (47).

The best results with alternating CAV and CDDP+VP16 have been published by the Memorial Sloan-Kettering Cancer Center Group (48).

Forty four patients were treated with the alternating regimen for 4 courses. Patients recycled after 2 weeks only if WBC count was over 3000/ul and platelets over 100.000/ul. The drugs were administered at 2 week intervals in 80% of patients. Thoracic radiotherapy and PCI were delivered to the patients with limited disease. Maintenance chemotherapy with alternating CMP (CCNU, Methotrexate and Procarbazine), CAV and DDP + VP16 where also delivered to all patients with extensive disease and with limited disease not reaching complete remission (CR).

An impressive 67% CR rate with a median survival of 18.5

months in limited disease patients and a 65% CR rate with a median survival of 12.2 months for extensive disease patients were obtained.

TABLE 6 - CAV ALTERNATING WITH CDDP + VP16

	PTS	CR	OR	MEDIAN SURVIVAL
LIMITED DISEASE				
1) FELD, 1985 (Randomized) q. 3 weeks-6 cycles. Toracic RT + PCI No maintenance (45).	105	53%	79%	14 mo.
2) GOODMAN, 1986 (Randomized) q. 3 weeks-6 cycles Toracic RT + PCI Maintenance (46)	200	49%	71%	17 mo.
3) NATALE, 1985 q. 2 weeks in 80% of Pts-4 cycles Toracic RT + PCI Maintenance (47)	24	67%	95	18,5 mo.
4) PACCAGNELLA, 1987 q. 3 weeks-4 cycles toracic RT + PCI Maintenance	30	33%	80%	14 mo.
EXTENDED DISEASE				
1) EVANS, 1986 (Randomized) q. 3 weeks-6 cycles PCI in responding Pts No maintenance (48)	123	40%	61%	9,8 mo.
2) NATALE, 1985 q. 2 weeks in 80% of Pts-4 cycles PCI in responding Pts Maintenance (47)	20	65%	95%	12,2 mo.
3) PACCAGNELLA, 1987 q. 3 weeks-4 cycles PCI in responding Pts Maintenance	55	15%	68%	9 mo.

In the attempt to reproduce the Memorial Sloan-Kettering results, our group adopted the same protocol with the only difference of recycling the alternating regimens during induction treatment every 3 weeks instead of 2 weeks. Our preliminary data on 85 evaluable patients (Table 6) show inferior complete and partial response rates and median survival with values comparable to those of the other studies recycling CAV and CDDP + VP16 every 3 weeks.

Since the Goldie and Coldman model predicts that the period in which the tumor is sensible to chemotherapy is short and that drug resistance occurs rapidly with each cell division, it is possible that the excellent results by Memorial Sloan-Kettering Study Group are, at least partially, due to the rapid recycling during the induction phase. Based on this assumption, a regimen has been designed (CDDP + DXR + VCR + VP16) with cycling of drugs at weekly intervals (49). Preliminary results in extensive stage SCLC showed 2CRS and 6PRS out of 8 patients.

The principal side effect was transitory myelosuppression; with the antimicrobial prophylaxis there were no infectious complications and the regimen was delivered on an outpatient basis.

NON SMALL CELL LUNG CANCER

The value of chemotherapy in NSCLC is controversial. Conflicting data from several phase II studies, the small rate of complete responders, the short response duration, the poor benefit in terms of improved survival and the drug toxicity, make difficult to realize large phase III studies to exactly define the role of chemotherapy in NSCLC.

The cumulative response rate of the most active single agents in NSCLC (except for platinum derivates) are listed in (table 7) (50, 51).

TABLE 7 - SINGLE AGENTS IN NSCLC (PLATINUM DERIVATES EXCLUDED))

DRUG	NO of PTS	RESPONDERS
IPHOSPHAMIDE	237	26%
MITOMYCIN	115	20%
VINBLASTINE	27	20%
VINDESINE	406	17%
ADRIAMYCIN	283	12%
ETOPOSIDE (VP16)	278	10%
METHOTREXATE	247	40%
CYCLOPHOSPHAMIDE	369	8%
CCNU	161	7%

Cisplatin as single drug showed a moderate activity. The cumulative response rate was 15%, with one CR only, out 310 treated patients. CDDP seemed mainly active in epidermoid and adeno-carcinoma (Table 8) (52).

Carboplatin tested in 70 patients with 3 different dose schedules showed no activity, producing one objective response (53, 54, 16). Similarly with DAACP Scher observed only one objective response in 31 tested patients (55).

Iproplatin appeared the only second generation platinum analog showing to be active at least as cisplatin with a 17% response rate (52).

TABLE 8 - PLATINUM DERIVATES IN NSCLC

	NO OF PTS	RESPONDERS PTS	%
CIS PLATIN 75-140 mg/m2 q. 3-6 weeks (52)			
EPIDERMOIDAL	170	29	17%
ADENO	161	17	10%
LARGE CELL	29	2	7%
TOTAL	310	48	15%
CARBOPLATIN			
1) OLVER 80 mg/m2 1-5 q. 4 weeks (53)	38	0%	
2) KREISMAN 400 mg/m2 q. 4 weeks (54)	26	1	4%
3) GORE (16)	6	-	0%
IPROPLATIN			
KREISMAN 270 mg/m2 q. 4 weeks (54)	24	4	17%
DACCP			
SCHER 640-720 mg/m2 q. 3 weeks (55)	31	1	3%

Combinations of traditional drugs in advanced NSCLC have shown no better activity than single agents when comparative randomized trials were carried out (56). When the most active

single drugs (Vindesine, VP16, Ifosfamide, Mytomycin) were combined with CDDP it was possible to obtain a sinergistic or an additive effect (Table 9).

TABLE 9 - EFFECTIVE REGIMENS IN NSCL

	PTS	RESPONSE RATE
CDDP + VP16	682	30%
CDDP + VINDESINE	277	36%
CDDP + VP16 + VINDESINE	326	28%
CDDP + MITOMYCIN + BLEOMYCIN	104	27%
CDDP + ADRIAMYCIN + CYCLOPH.	131	27%

Cisplatin containing combinations led, in a series of randomized studies, to a 30-45% response rate with 5% of CR (57). The most active regimens combined CDDP and VP16 and CDDP and Vindesine (51).

Comparative studies demonstrated the superiority of CDDP + VDS over VDS alone (61, 64, 65).

No randomized studies tested VP16 alone versus CDDP+VP16 in NSCLC.

Many clinical trials tested CDDP containing combination against regimens without CDDP.

A significant advantage in response rate was generally found in favour of the CDDP containing combinations (62, 63, 65, 60, 52).

Our group tested in a randomized study CDDP+VDS and CDDP+VP16 versus Doxorubicin + Cyclophosphamide as control group.

Although the CDDP containing regimens showed a significantly superior response rate (48% vs 36% vs 10% respectively) the median survival was similar for the three groups (43 mo. 47 mo. and 41 mo.) The lack of favourable effects of the two CDDP regimens on survival is probably related to the short response duration that is generally observed in NSCLC (66).

The CDDP containing regimens with more than one active drug (VP16, VDS, Mitomycin) seemed not superior to the combinations with CDDP plus only one drug, both in terms of response rate and response duration (58, 59, 64).

Recently the EORTC Lung Cancer Working Party reported the results of a large randomized study comparing high (120 mg/m^2) and standard (60 mg/m^2) doses of CDDP in combination with VP16. The objective response rate was 25% in the standard dose arm and 29% in the high dose arm. No significant differences in response duration median survival of responders and overall survival (67) was shown.

Gandara obtained 38% of responses in advanced NSCLC patients with a modified high dose cisplatin (200 mg/m^2) schedule (68,69).

The problem of a dose-response relationship for CDDP in NSCLC remains however open.

Up to now chemotherapy has been mainly used in NSCLC for palliation of patients with advanced, symptomatic, disease.

Since the response rate was generally less than 50%; the response duration short (3-5 months) and the toxicity considerable, many Authors concluded that supportive care only was the best strategy for this type of patients. In addition the patients with more chance to respond to chemotherapy were the less symptomatic.

A NCI-C trial compared best supportive care to two cisplatin containing regimens in the management of advanced NSCLC (70). Patients submitted to CDDP + VP16 obtained a 25% response rate only but the median survival of patients was nearly doubled when compared to supportive care alone.

In conclusion the role of chemotherapy in advanced patients with NSCLC remains to be defined; quality of life studies for patients submitted to cytotoxic therapy or followed with supportive therapy only are needed.

Preliminary results of trials using chemotherapy in an adjuvant

setting for operable NSCLC patients seem promising (71). In the future the combined modalities approach should be investigated under appropriate clinical conditions.

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PLATINUM, THE SYNERGISTIC DRUG: FROM CLINICAL EVIDENCE TO LABORATORY SUGGESTIONS

M.V. Fiorentino and C. Ghiotto

INTRODUCTION

Clinical or "therapeutic" synergism, requires a complex balance of therapeutic and toxic interactions.

Simple additivity of benefits, without the same additivity in side effects, represents a therapeutic advantage, probably more acceptable than true synergism of the therapeutic actions, accompanied by summation or synergism of the toxic effects.

The term "synergism" applied to this situation, although incorrect may be operationally useful.

Sometimes, even the summation of "different" side effects (example giving, moderate alopecia from drug A + moderate leucopenia from drug B) may be acceptable, comparing with potentiation of one side effect, e.g. when moderate leukopenia related to drug C or to drug D, may result in definitive agranulocytosis from the combination of drug C and D.

From a clinical point of view we will compare, for specific tumours, couples of regimens differing only for presence or absence of Platinum (Pt).

When the general cumulative toxicity of each regimen remains within ethical and psycological acceptability we will compare the results in terms of Complete Remission (CR) and Partial Remission

(PR) or Overall Remission Rate (CR+PR) and - when appropriate - of remission duration, or cure rate.

TOXIC SYNERGISM

When combining Pt with other drugs, special attention should be paid to toxic synergism.

From a theoretical point of view it is easy to preview that the renal clearance of Methotrexate given after Pt may be hindered, with increased exposure of tissues and increased overall effectiveness and toxicity.

Bleomycin, given concomitantly or before Pt, may impair the Pt disposal through the kidney and increase at least nephrotoxicity, but often may as well increase the general toxicity from Pt.

Vincristine may sum its own neurotoxicity when added to Pt, and deafness, peripheral neuritis and palsies may appear more frequently and in a heavier form. It is well apparent that the development of prohibitive levels of the previously described toxicities may prevent the administration of therapeutically synergistic combinations of those drugs.

High doses of Pt will also increase nausea and vomiting from anthracyclines and Dactinomycin or cause unacceptable myelotoxicity also from Cytoxan and Fluorouracil.

A special note on cardiac toxicity frequently occurring with the Wayne University regimen for Head and Neck malignancies is due; in our experience a reduction of the Fu doses and of the duration of the treatment course has often proven necessary.

Platinum induced hypomagnesemia is also more frequent and more pronounced when other cytotoxics are given concomitantly (Cytoxan; Velban; Antracyclines, Bleomycin) according to our experience.

LUNG CANCER

For Lung Cancer the Small Cell type has a response rate to Pt as single agent ranging from 0% (1) to 9 or 12% (2, 3).
Pt has been studied in combination with a number of other chemotherapeutic agents in L 1210. Evidence of synergism of Pt has been noted for some of these other agents, including Cyclophosphamide, Iphosphamide, Vincristine, Adriamycin, Etoposide and Methotrexate, all agents possessing some clinical activity in lung cancer (26)(65).

According to preclinical findings, Evans (4) administered Pt+ Etoposide (Et) + low dose radiation to the primary in 28 evaluable patients obtaining an 86% OR rate (43% CR) while Murray alternating the Pt Et regime with CAV (Cyclophosphamide, Doxorubicin and Vincristine) obtained 94% OR, 70% being complete responders among 67 patients (5) and the whole group reaching a projected 2 year survival of 35%.

A clinical synergism is at least suggested in this case.
Synergism with radiation has recently been shown expecially in cell cultures (6) with repair inhibition of DNA damage.

In the Non Small Cell type of lung cancer, with Pt alone, on 38 patients there have been only 3 responses (approx 8%) (1)(5)(7). It is known that Vindesine (VD) alone (8) may show some activity ranging from 20% of patients to 8% (9) In our experience 116 patients with advanced non small cell carcinoma of the lung, were randomly assigned to 3 combinations as follows : Pt+ Vindesine (VD), Pt plus Et and Doxorubicin plus Cyclophosphamide. 94 patients were evaluable for response. Of 31 pts, 15 (48%; 3 CR and 12 PR) responded to Pt + VD; of 33 pts, 12 (36%; 2 CR and 10 PR) responded to Pt + Et; of 30 pts, 3 (10%) obtained a PR with Doxorubicin + Cyclophosphamide (11).

The combination of Pt and VD seems to have increased the response rate from a maximum expected of 28% to an observed 48% (10) (9). If these optimistic data as ours are to be accepted, the term synergism may be appropriate for NSCCL, on the basis of the response rates obtained; but the best regimens are followed only by an increased survival of "responders"; not by an increased overall survival of the "treated patients".

This association (VD+Pt in NSCCL) has not had origin from in vitro or animal data; it is concievable that appropriate experimental studies may suggest how to increase its effectiveness.

A number of relatively active and more extensively tested regimens have included Pt combined either with a vinca alkaloid (12) (14) (16) or with etoposide (13) (15). The initial response rates reported for "Pt" and "non Pt containing" regimens ranged from 28% to 53% (13) (15). However, subsequent evaluation of each of these regimens in trials of the Eastern Cooperative Oncology Group (ECOG), involving larger cohorts of patients has shown response rates of 12% and 17% for the non Pt containing regimens, and 23% to 31% for the Pt regimens (15).(See tab.1)

Although the rr of these combination regimens, ranges from 25 to 53% in these trials (Table 1) there is no consistent trend of a significant difference.

Table 1. Pt regimens in Non Small Cell Lung Cancer

Regimen	Response Rate (%)	References
Pt + VD	43	(12)
Pt + VD	25	(12)
Pt + Et	28	(13)
Pt + Et	25	(15)
Pt + VB + Mit.C	53	(14)
Pt + VB + Mit.C	31	(16)

VD = Vindesine
ET = Etoposide
Mit.C = Mitomycin-C
VB = Vinblastine

BLADDER

At present time there is no curative chemotherapeutic regimen for this type of tumour.

Pt used as single agent induces a remission of the disease in 37 to 45% of the patients (17)(18).

Furthermore, Pt was used in combination with other chemotherapeutic agents, including Cyclophosphamide and Doxorubicin. (See Tab.2).

Table 2. Pt single agent and Pt in association in bladder cancer.

Drugs	% Overall Response	References
Pt	37%	(17)
	45%	(18)
Pt + Cy	50%	(19)
Pt + ADM	50%	(20)
Pt + Cy + ADM	50%	(21)

As one can see from Table 2, even if a higher response rate has been shown when Pt (19)(20)(21), was combined whit Cy or ADM, the role of these associations has not been defined.

Important results have been achieved by Sternberg (21) on 64 patients with metastatic bladder cancer, with a combination inclusive of Methotrexate, Velban, Doxorubicin and Pt. The objective response rate was 70% with 39% CR and 31% PR. Although the CR have been pathologically confirmed, unfortunately relapse and/or disease progression usually occurs after the third course of therapy.

So we can only conclude that Pt as single agent or in association is an effective drug in bladder carcinoma.

HEAD and NECK

For Head and Neck (epidermoid) cancers, before the introduction of Pt containing combinations the potential of chemotherapy was very low: Methotrexate (22) and Bleomycin were among the most active single agents, obtaining short lived responses in approximately 20% of patients, while 5-Fluorouracil was barely half as effective. Pt alone matched these results at low doses and quoted around 30% at higher doses (See tab. 3).

Several combinations of Pt with Methotrexate, Bleomycin, Adriamycin and Vincristine (Tab. 3) brought the response rate around 60% (averaging several reports).

The combination of Pt and Fu (where simple additivity would yeld around 30%) has consistently given 94% overall and 63% complete remission to Deker (23) and, in non pretreated patients, 84 and 27% to our group (24).

In this case true synergism is strongly suggested, and the probability of synergism had been a priori derived from in vitro

experimental studies (25)(26).

Table 3. Effective combination in HN tumours

Regimen	N° Pts	%OR	References
Pt + Fu	70	84	(24)
Pt + Fu	35	94	(23)
Pt + Bleo	28	11	(27)
Pt + ADM	10	50	(28)
Pt + Mtx	20	60	(29)
Pt + Mtx + Bleo	37	51	(30)
Pt + Mtx + Bleo	11	55	(31)
Pt + Bleo + Mtx	19	75	(32)
Pt + Bleo + Vcr + Mtx	22	27	(33)
Pt + Vcr + Bleo	10	50	(34)

Our concern on cardiac toxicity of the first combination has been cited above and possibly this side effect is linked more with the modalities (24 hr infusion of 5-Fu) than with the Pt + Fu combination.

OESOPHAGEAL CANCER

In oesophageal cancer no single drug has consistently given a response rate over 15%, including Pt, Fu, Bleomycin, Doxorubicin and Vindesine.

The first 2 drugs of this list (Pt+Fu) when combined have obtained up to 80% responses in small series of patients with non metastatic tumor (35).

In advanced disease the response rate ranges between 33 and 50% according to various reports (36) (37) (38) (39).

Also in this case synergism more than simple additivity is strongly suggested by clinical finding, in agreement with preclinical

researches (25)(26).

In our first experience using Pt along with Methotrexate and Bleomycin, a short lived 26% response rate was observed. Since 1983 we used Pt combined with 5 Fu (40). 6 complete and 16 partial responses on 61 patients were seen, for a total response rate of 36%. Duration of response was 7.5 (2-48) months. Our results seem to confirm the efficacy of the Pt+ Fu combination and encourage the use of such treatment not only as an adjuvant but also in a neoadjuvant setting.

BREAST CANCER

The usefulness of Pt as single agent in breast cancer was tested by several Authors.

Kolariç with Pt alone (30 mg/m^2 on 4 consecutives days) in non pretreated pts reported an overall response rate of 54% (19/35 pts) with 13 CR (37%) and 6 PR (17%)(41).

Pt used as single agent in second line therapy, does not seem to be so effective (Yap, 42).

Kolariç was the first to employ Pt in combination; in that schedule Pt (30 mg/m^2 days 1;3 and 5) was associated with Cytoxan (200 mg/m^2 days 1;3 and 5) and with Adriamycin (40 mg/m^2 in day 1) (CAP 5). For non pretreated pts with disseminated breast cancer he reported an objective response in 43/61 pts (72%) with 36% CR (43).

The response rate seems not influenced by menopausal status, praevious radiation or hormonal therapy.

With the same schedule as second line therapy in patients pretreated with CMFVP (Cytoxan, Methotrexate, 5-Fluorouracil and Prednisolone), the same Group reported an objective response in 20/39 pts (51%).

In order to demonstrate the effectiveness of Pt in breast

cancer, Kolariç recently (44) compared the CAP 5 schedule vs FAC (5-Fu, Doxorubicin and Cytoxan). With CAP 5 the overall response was 58% with a good CR rate (32%), whereas FAC gave a 47% Overall Response with only 12% CR.

A study of ours confirms a 47% objective response rate in pretreated mammary cancer with the CAP 5 regime (45). Effectiveness of CAP is good; synergism not demonstrated.

TESTIS

In testicular cancer the combination of Pt with Bleomycin + Velban or Etoposide may cure around 70% of patients. The last drugs of the combination are effective in 10 to 20% of the patients when used alone, or combined without Pt.

No preclinical data suggested this association. The activity of Pt alone appeared in one report of 11 cases (with 8 OR)(46). The Authors combined Pt with Velban + Bleo, being these two drugs the more fashionable combination of that time.

No further assessment of Pt alone has been performed and consequently the famous effectiveness of the PVB combination may indeed be attributed to Pt alone and synergism may not be claimed.

THYMOMA

Invasive and or metastatic thymoma was considered a chemoresistant tumour, although very small series had been treated. The value of Pt in metastatic thymoma was firstly noted in a case report by Talley (47) with high dose Pt (100 mg/m^2) with an excellent objective response

Other single observations in a multi-drug regimen have been reported by Cocconi (48) and, by Campbell (49). These responses lasted 10 or more months.

Basing on these preliminary reports we set up a Pt containing program (50 mg/m^2 day 1) in combination with Doxorubicyn (40mg/m^2 day 1) , Vincristine (0.6 mg/m^2 day 3) and Cyclophosphamide (700 mg/m^2 day 4) with the acronym ADOC. Of 27 patients with invasive thymoma an objective response was reached in 91% ; 40% obtained a Complete Remission (50; Fiorentino et al., in press.).

Althoug the ADOC regime seems very effective no assessment of "synergism" is allowed, because Pt used alone seems "per se" active, and its activity has not been appropriately measured.

OVARY

Pt is known as one of the most active agents in ovarian cancer and was used in different combinations and clinical situations.

Pt as single agent

Low doses:

the effectiveness of Pt at low doses (30-50mg/mq every 3-4 weeks) was tested by some Autours with poor results (see tab. 4)

Table 4. Pt low doses in ovarian cancer

RO/Pts	% RO	References
4/18	22%	(51)
6/19	31%	(52)
9/37	24%	(53)
5/19	26%	(54)

High doses:

Pt at high doses (100-120mg/mq) was used as first line therapy by Wiltshaw, who reported an overall response rate of 52.3% (11/21 patients)(54). Using the same doses Barker (55) reports an overall

response of 55% (17/31) in patients refractory to alkylating agents. Furthermore he reports 2 responses over 9 patients pretreated with low doses Pt (22%).

Piver et al. (56) instead, reported unsatisfactory results from high dose Pt in heavily pretreated patients (only 1 response over 20 patients).

Pt in association with other chemotherapeutic agents:

Several Pt containing combinations have given favourable results in cancer of the ovary: see table 5.

Table 5. Results of Combination Chemotherapy in Advanced Ovarian Cancer

Regimen	Pts	OR (%)	References
H + Cy + ADM + Pt	51	28 (75)	(57)
Pt + ADM + Cy	56	44 (79)	(58)
ADM +Cy	41	35 (83)	(59)

H = Hexamethylmelamine
ADM = Adriamycin
Cy = Cyclophosphamide

Pt + Cyclophosphamide or Pt + Adriamycin or the triple combination PAC are almost equally effective in this disease and no specific synergism can be distinguished.

Alternating regimes inclusive of 5 to 7 drugs do not increase the effectiveness.

In our view the main advantage of Pt containing regimes is to show effectiveness also as second line treatments, in patients pretreated either with Hexa CAF, ACy or with other non Pt regimes.

OSTEOGENIC SARCOMA

Winkler (60) published the results of a multiple chemotherapy neoadjuvant trial.

All patients received high dose Methotrexate and Doxorubicin, either with Pt (for B group of patients), or (in group A) with a triple drug combination: Bleomycin, Cyclophosphamide and Dactinomycin (BCD). All patients were additionally randomized to receive fibroblast interferon versus nil (See tab. 6)

Table 6.

Drugs			2-year DFS
Mtx Doxo	BCD	+ IFN	85%
		- IFN	70%
Mtx Doxo	Pt	+ IFN	78%
		- IFN	79%

The disease free survival for the BCD and Pt group is exactly the same.

CERVIX

The demonstration that Pt is the single most active drug for chemotherapy of advanced squamous cell cancer of the uterine cervix (61)(62) opened the way to regimens combining this agent with others of known activity.

Vogl (63) and Alberts (64) used in advanced disease (III and IV Stage) slightly different combination chemotherapy regimens containing Mitomycin C, Vincristine , Bleomycin and Pt (BOMP) and

both obtained a good response rate (see table 7)

Table 7. BOMP regimens in Cervix Cancer

	Albert D.S.		Vogl S.E.	
	mg/m^2	days	mg/m^2	days
Bleomycin	20	1 to 4	10	weekly
Vincristine	0.5	1, 4	1	weekly
Mitomycin-C	10	2	10	1
Pt	50	1, 22	50	1, 22
recicle every	6 wks		6 wks	
cases treated	14		13	
overall RR (CR)%	50(36)		78(25)	

Our clinical experience confirms the efficacy of these regimens but possible synergistic conditions are not proven.

NEW PERSPECTIVES WITH BIOLOGICAL RESPONSE MODIFIERS

Special possibilities arise in the association of Platinum with Biological Response Modifiers like the Interferons.

In particular alfa Interferon (IF) has been tested against ovarian cancer in vitro and in vivo (66/67) with the clonogenic assay. The effect of growth retardation is not regularly dose-dependent as 100 units/ml of I.F. may potentiate the effect of Pt more than 1000 u/ml, obtaining a reduction to 14.3% of the in vitro growth (66; Welander cit by Smith)).

For lung cancer the trend is similar in various non small cell

types, although it is more prominent in the adenocarcinoma variety: when this tumour is grafted in nude mice the growth delay is 0.36 with Cisplatin; 0,64 with Cyclophosphamide and 0.91 with I.F. The combination of IF + Pt obtains a growth delay of 3.18 doubling times (68).

This data have been confirmed in various tumour lines, with Lymphoblastoid as well as with alfa2 recombinant I.F. : "strong evidence of a positive or synergistic interaction" has been found (69).

Lines of osteosarcoma neuroblastoma and CCL have given similar results to **Dannecker** (70).

Both Pt and Intron A given intraperitoneally are advocated by Welander for ovarian carcinoma with minimal residual disease (71).

In vitro Treuner (72) in lines of osteosarcoma or neuroblastoma found synergism with Pt and VP while Mowshowitz (73) has got contrasting results on the P388 leukemia.

A new field of combination of platinum with agents free from similar and/or cumulative toxicity is accordingly open for clinical promising applications.

DISCUSSION

Experimental studies suggested that Doxorubicin, Methotrexate, Etoposide Ara-C and 5-Fu could be at least additive or possibly synergistic with Pt. Other combinations could be studied however with transplanted and/or cultured tumours.

We suggest to revert from the clinical to the experimental field in order to optimize, in term of cronology and dose, some combinations already shown to be effective in the clinical setting.

A series of tests where Pt and the proposed synergistic agent or agents can be administered either concomitantly or in different sequences can be performed on small animals or on vitro cultures.

After having established the optimal sequence, we can find the minimum effective doses, or the optimal proportions between 2 or more agents.

A ping-pong movement of the research impetus between Pharmacologists and Clinicians may represent a source of outstanding progress.

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CHEMOTHERAPY OF MAMMARY CANCER WITH ANTHRACYCLINS AND ALKYLATORS

K. Kolarić

Over the past two and half decades the chemotherapy of disseminated breast cancer has involved three major stages. The first was single agent therapy. In 1969 Cooper (1) introduced polychemotherapy with the still "sacrosanct" combination of five drugs (Cyclophosphamide, Methotrexate, 5-Fluorouracil, Vincristine and Prednisolone; CMFVP). The third major stage in the development of breast cancer chemotherapy has been distinguished by the addition of Doxorubicin , one of the most active single agents in the tumor under consideration. What has Doxorubicin actually contributed? Controlled trials with Doxorubicin -containing versus Doxorubicin -non-containing regimens have shown, more rapid remission,a somewhat higher overall response rate but not at a statistically significant level, and a somewhat better response in visceral organs (lungs in particular). However, the duration of the remissions and the survival rate, as compared with the CMFVP regimen, have remained the same. The introduction of some new cytostatic drugs, i.e., Mitoxantrone (with a lower cardiotoxicity, but also a somewhat lower antitumorigenic effect as compared to Doxorubicin) and Epidoxorubicin (lower cardiotoxicity with the same antitumorigenic effect), has not brought about - apart from lower toxicity - a marked improvement in breast cancer chemotherapy. This has even less been the case with Vindesine, Vinblastine, Dibromdulci-

tol, Hexamethylmelamine, Mitomycin C or VP-16, the antitumorigenic effect of which lags considerably behind that of the five standard cytostatic drugs (Doxorubicin , Cyclophosphamide, 5-Fluorouracile, Methotrexate, Vincristine) currently applied in the treatment of breast cancer.

Cis Platinum, a widely used cytostatic agent, whose differential cytotoxic activity may be related to differential ability to cross link DNA strands having a similarity to byfunctional alkylating agents, sofar was not studied sufficiently in breast cancer. In accordance with in vitro and in vivo findings, synergistic antitumor activity of Cis Platinum has also been detected with variety of cytostatic agents but particularly with Cyclophosphamide, Adriamycine and VP-16. If one considers the fact that the antitumorigenic effect of Cis Platinum is highly dose-dependent, it is rather surprising that the results obtained in their controlled randomized trials by Hakes and Forastier (1979; 1982) - who tested by Phase II trials the effect of low vs. high Cis Platinum doses (60 mg/m^2 vs. 120 mg/m^2) in previously heavily pretreated patients - have not stimulated further studies (2,3). We are emphasizing this point because, these high doses produced regression in 29 and 21 %, respectively, of treated patients, and it is well known that with such results in previously untreated patients we may expect a response in at least 35-50 % cases. Thus until our trial, Cis Platinum had not for all intents and purposes been tested in untreated patients, and the established view, - still found in every oncological journal and textbook - has been that Cis Platinum is an inactive drug in breast cancer. It is not our intention to hypothesize why Cis Platinum has not been adequately tested in breast cancer as well, although - as already mentioned - many objective reasons would support

its use in this disease as well. Therefore, we were surprised - while testing in a Phase II trial the anti tumorigenic activity of Cis Platinum in metastatic brain tumors (1982) - when we, for the first time observed an antitumorigenic effect of the drug in primary tumors and extracerebral breast cancer metastases (4). Our patients had been administered no prior cytostatic drug therapy, and we explained the effect by this factor. On the occasion, we observed objective remissions in 5 out of 12 patients, three remissions having been complete. These facts have been the rationale for a series of studies we have performed with this compound in breast cancer, starting with a Phase II trial in untreated patients (30 mg/m^2 daily x 4). The trial, the results of which were published in 1983, demonstrated the high antitumorigenic activity of Cis Platinum in a group including 35 patients (the response rate was 54 %, with a high percentage, 37 %, of complete remissions), which is rather rare in breast cancer (5), but having in mind that 19 out of 35 evaluable patients had the predominant metastatic site in soft tissue considered to be a more favourable prognostic factor. The most favourable response was observed in soft tissue - 68 % - whereas the response in visceral organs was 40 %. Except for pronounced pain relief, there was no objective regression as far as bone alterations were concerned. These results showed that the antitumorigenic effect of Cis Platinum matches that of Doxorubicin - the optimum cytostatic drug in breast cancer. Toxic side effects, mainly bone marrow suppression (particularly anemia- 68 %) were moderate and tolerable.

Our observations provided the rationale for the integration of Cis Platinum into combination chemotherapy (Phase III trial) with Doxorubicin and Cyclophosphamide (CAP), i.e., cytostatic drugs with which

Cis Platinum has been shown to produce a marked synergistic effect in both animal and human tumors. To be sure, our regimen (Cyclophosphamide 200 mg/m^2 i.v. on days 1,3,5; Doxorubicin 40 mg/m^2 i.v. on day 1; Cis Platinum 30 mg/m^2i.v. on days 1,3,5 - 90 mg/m^2/per cycle) differed significantly, as far as the dose was concerned, from the frequently used one-day CAP regimen. Our report on the trial was published in 1985, and in it we compared CAP with CMFVP (6). Platinum-containing chemotherapy offered significant advantages in terms of the overall response (72 % vs. 42 %), complete response (36 % vs. 16 %), and the response in soft tissues and visceral organs (50 % vs. 41 % and 58 % vs. 33 %, resp) (Table 1).

Table 1. Results of CAP vs. CMFVP combination chemotherapy in metastatic breast cancer

Schedule	No. of evaluable patients	CR	PR	SD	PROG	Objective response
CAP	61	22 (36 %)	21	8	10	43/16 (72 %)
CMFVP	62	10 (16 %)	16	9	27	26/62 (42 %)

Objective response: $P < 0.01$
Complete response : $P < 0.01$
Legend: CR - complete response; PR - partial response; SD - stable disease; PROG - progression

The differences with regard to the median duration of remissions (16 vs. 9 months) and the survival rate (two-year disease-free period in 53 % of patients on CAP vs. 33 % in patients on CMFVP) was also statistically significant ($P < 0.01$). In this way the marked advantage of Cis Platinum-containing chemotherapy over the CMFVP regimen was clearly demonstrated. Concerning

toxicity, side effects were more pronounced in CAP group, particularly myelosuppression with anemia prevailing. However, no drug related deaths were observed.

Within the scope of the same trial the patients showing primary resistance or disease recurrence after the CMFVP regimen were administered CAP as a second--line therapy. The result was an unusually high overall response, i.e., 51 % (20/39) with no fewer than 7 complete remissions (18 %), which is rather unusual for second-line therapy in breast cancer (Table 2).

Table 2. CAP combination chemotherapy as a second-line treatment in patients with CMFVP failure

Predominant metastatic site	CR	PR	SD	PROG	Objective response (CR + PR)
Soft Tissue	2	7	1	2	9/12 (75 %)
Visceral organs	5	6	2	7	11/20 (55 %)
Bones	-	-	-	7	0/7
Total	7	13	3	16	20/39 (51 %)

Legend: CR - complete response; PR - partial response; SD - stable disease; PROG - progression

The results we have observed in CAP vs. CMFVP study led us to the practically most important question - we had asked ourselves even earlier, i.e., whether is CAP, as a first-line treatment, superior to a Doxorubycine-containing regimen? Designing the new Phase III trial we opted for FAC regimen (5-Fluorouracil, Adriamycine, Cyclophosphamide), because this combination does, after all, produce the most constant rate of objective remissions (50 - 70 %).

During the period from June 1985 till June 1986 142 patients entered the study. Out of 126 evaluable patients (> 2 cycles) with an almost identical balance of prognostic factors in both treatment modalities, 58 were on the CAP and 68 on the FAC regimen (Table 3).

Table 3. Patient characteristics

	Total No. of patients	CAP	FAC
No. of patients entered the study	142	68	74
Evaluable patients (> 2 cycles)	126	58	68
Age (years)	29 - 70	29 - 70	35 - 70
Median	53	51	54
Premenopausal patients	35	20	15
Postmenopausal patients	91	38	53
Previous radiation	87	44	43
Previous hormone therapy	28	14	14
Performance status (Karnofsky scale)			
40 - 70	47	19	28
80 - 100	79	39	40
Predominant metastatic site			
soft tissue	53	28	25
visceral organs	52	21	31
bones	21	9	12

The overall response rate was 67 % for CAP (35/58) and 41 % for FAC (28/68) - $P < 0.005$. The difference was also statistically significant - $P < 0.05$ - for the complete remission rate (CAP: 26 %, 15/58; FAC: 12 %, 8/68). (Table 4).

Table 4. CAP vs. FAC combination chemotherapy in metastatic breast cancer

CAP	No. of evaluable patients	CR	PR	SD	PROG	Response rate
Soft tissue	28	8	14	4	2	22/28 (78%)
Visceral organs	21	7	8	5	1	15/21 (71%)
Bones	9	-	2	6	1	2/9
Total	58	15 (26%)	24	15	4	39/58 (67%)
		P < 0.05				
FAC						
Soft tissue	25	3	11	7	4	14/25 (56%)
Visceral organs	31	5	8	10	8	13/31 (42%)
Bones	12	-	1	7	4	1/12
Total	68	8 (12%)	20	24	16	28/68 (41%)

CAP Soft tissue + visceral organs:
Overall response 75 % (37/49) CR 31 % (15/49) $P < 0.005$

FAC Soft tissue + visceral organs:
Overall response 48 % (27/56) CR 12 % (8/56)

Legend: CR - complete response; PR - partial response; SD - stable disease; PROG - progression

In the CAP group a high response rate was noted in soft tissues (78 %, 22/28) and visceral organs (71 %, 15/21), as compared with the FAC regimen (soft tissues 56 %, visceral organs 42 %). Where bone lesions are concerned, there were no differences. Although no differences have

been observed, for the time being, with regard to the median duration of remission (CAP M= 12+ months as compared to FAC M= 11+ months), the difference between the survival rates as preliminarly observed, was statistically significant (CAP M= 18+ months as compared to FAC M= 14+ months; $P < 0.05$). To date our patients have been followed-up for an average period of 18 months (12-24). Insofar as toxic side effects are concerned, no major differences were noted except for more pronounced bone marrow suppression in the CAP group (67 vs. 37 %). (Table 5).

Table 5. Toxic side effects

	CAP 58 patients	FAC 68 patients
Anaemia		
Grade I	16	7
Grade II	10 (46 %)	3 (15 %)
Grade III-IV	1	-
	67 %	37 %
Leukopenia		
Grade I	6	7
Grade II	5 (21 %)	3 (22 %)
Grade III-IV	1	5
Thrombocytopenia	-	-
Creatinine		
Grade II	1	-
Neurotoxicity	3	-
Ototoxicity	-	-
Nausea-vomiting		
Grade I	5	25
Grade II	27	12
Grade III-IV	23	4
Alopecia		
Grade I	2	4
Grade II	14	21
Grade III-IV	37	41
Stomatitis		
Grade II	1	-
ECG Changes	1	2
CHF (ADM - 350 mg/m^2)	-	1

Cis Platinum did not cause any serious neuro or nephro--toxic complications either. Accordingly, our latest trial has demonstrated the advantage of CAP chemotherapy even over Doxorubicin -containing regimens. Since this is the first trial of this kind, the role of Cis Platinum in first line combination breast cancer chemotherapy calls for additional clinical studies, although it is already much more obvious that Cis Platinum is truly a useful and active drug in breast cancer chemotherapy, what is now also reconfirmed by the other authors.

Namely, very recently (1987) Einhorn and associates in a Phase II single agent trial (120 mg/m^2 per cycle) in previously untreated patients observed a 45% response rate (9/20) with median remission duration of 4,5 months. Six partial responses were observed in liver and lung and three in soft tissues. Yet in 1984 Mechl also reported 47 % response rate in a small group previously untreated patients (5/12) (8). Summarizing Phase II Cis Platinum studies in breast cancer (120 mg/m^2 per cycle), an average of 49 % response rate (33/67) with 16 % complete remission rate was observed, thus there is no doubt that Cis Platinum is one of the very active agents in breast cancer.

Concerning CAP regimen as a primary treatment in disseminated breast cancer, our results have recently been also reconfirmed by several groups. Thus CMEA Study Group in a Phase III trial comparing CAP vs. CMFVP observed 62 % response rate (54/87) with CAP and 43 % (32/82) with CMFVP regimen ($P < 0.005$) (9). In comparison to our study, there was no difference in remission duration (6 vs. 5,6 months), while it is also too early to make a decision regarding survival. Furthermore, Colozza et al. in a Phase II study also reported 61 % response rate (20/36) with 12 % CR, but the dose of Cis DDP in CAP regimen was decreased on

60 mg/m^2 per cycle. Nearly half of the patients have had previous CMF adjuvant treatment (10).

Remarkable results with CAP protocol, but as a second or third line treatment in refractory breast cancer were recently noted by many investigators (Table 6).

Table 6. Cis Platinum containing combination chemotherapy in refractory breast cancer (second and third line treatment)

Author	Schedule	No.of eval. pat.	Response rate (CR + PR)	CR	RD	DDP dose per cycle
KOLARIĆ 1984	CAP	39	20/39 (51%)	18%	7	90 mg/m^2
FIORENTINO 1986	CAP	36	17/36 (47%)	11%	6	90 mg/m^2
CARTEI 1986	CAP	54	27/54 (51%)	15%	4	90 mg/m^2
MECHL 1985	CAP	13	6/13 (47%)	14%	6	90 mg/m^2
JACOBS 1985	DDP+5-FU	14	5/14 (36%)	0	4	100 mg/m^2
GONZALES 1986	DDP+5-FU	16	11/16 (68%)		7	100 mg/m^2
BLUMEN-SCHEIN 1985	DDP+5-FU+ CPM+MTX	28	14/28 (50%)	0	6	60 mg/m^2
ISRAEL 1986	DDP+ADM+ BLM	30	21/30 (70%)	10%	10	100 mg/m^2
COCCONI 1986	DDP+VP-16	24	4/24 (17%)	0	4	80 mg/m^2
CALGB 1986	DDP+VP-16	42	7/42 (17%)	0	3	100 mg/m^2
COX 1987	DDP+VP-16	30	12/30 (37%)	9%	6	100 mg/m^2
BISAGNI 1986	DDP+VP-16	27	5/27 (16%)	4%	5	100 mg/m^2
	DDP+VP-16	26	2/26 (8%)	0	4	60 mg/m^2

Legend: CR - complete response; RD - remission duration (M= months)

Fiorentino and his group observed a response in 47 % of patients (17/36) with 11 % complete responses, while Cartei in the largest series registered 51 % responses (27/54) with 15 % complete responses (11,12). Twenty two patients had Adriamycine in previous chemotherapy. Particularly high response was observed in soft tissue (71 %), lung (62 %), and liver (40 %). Cartei considers the CAP regimen to be one of the truly optimal combined second or third line chemotherapies in disseminated breast cancer. It is necessary to mention that the common in all these studies was the dose of the drugs we proposed in CAP regimen (Cis Platinum 90 mg/m^2, Adriamycine 40 mg/m^2, Cyclophosphamide 600 mg/m^2 per cycle).The table shows also the results observed with the other Platinum-containing combination therapies. Particularly encouraging results have been observed with Cis DDP + 5-FU combination (Gonzales, 68 % responses - 11/16), than with Cis DDP + Adriamycine + Bleomycine combination (70 % response rate - 21/30, Israel) (13,14). Blumenschein, however, reported 50 % responses in 28 patients with Cis DDP, 5-FU, Cyclophosphamide, Methotrexate combination chemotherapy (15).

Particular attention should be paid on Cis Platinum + VP-16 combination in refractory breast cancer. Namely, the combination of Cis Platinum and Etoposide, which has shown an extraordinary synergistic effect both in animal and human tumors (ovaries, microcellular lung cancer, testes), does not appear to produce a significant effect when applied as second-line therapy in breast cancer. This view is supported by the reports of Cocconi (response rate of 17 % -4/24), as well as by the Phase II trial run by Cancer and Leukemia Group B where the same combination produced the response rate of 17 % (7/39) (16,17). It is nevertheless a fact that in 76 % of patients, in the last group, the predominant site of the disease were visceral organs, which is a

markedly poor prognostic factor. However, only one report with this combination was encouraging. 1987 Cox reported a response rate of 37 % (12/30) with 9 % of CR (18). Interestingly enough, it was precisely Cocconi et al. who in 1986 reported on a randomized trial involving two different Cis Platinum dose regimens (60 mg/m^2 vs. 100 mg/m^2) in combination with the same doses of Etoposide. Although the difference with regard to the therapeutic effect was not statistically significant, the higher Platinum dose did produce twice as many remissions (16 vs. 8 %) (19). To be sure, the trial involved third-line treatment of disseminated breast cancer, but Cocconi was nevertheless able to prove that the response depended mainly on the Cis Platinum dose. For the time being there is no explanation why the Cis Platinum - Etoposide combination has not proved to be as efficient as second-line therapy in the treatment of breast cancer. At any rate, Etoposide is not a drug of choice in the treatment of breast cancer.

Considering all these results, we believe that there is no doubt about CAP combination chemotherapy in accordance with the regimen we used - being an optimum first and second-line chemotherapy in disseminated breast cancer.

There is, however, a very complex question: what to do after a relapse, or after the development of primary resistance to first-line CAP combination chemotherapy? In our experience, there is as yet no effective second-line therapy after CAP, which practically leaves only hormonal therapy - unless it has not been applied already. Because of this point, future trials should also have to focus on the study and definition of those indications and subset of patients where the CAP regimen is irreplaceable in breast cancer treatment. Namely, perhaps one should not reject the idea whereby non-anthracycline-containing regimens (CMF, CMFVP, etc)

as first-line treatment, and CAP as second-line treatment, could possibly provide the most efficient (for the time being) approach to the treatment of disseminated breast cancer. To conclude, we believe that the introduction of Cis Platinum in breast cancer chemotherapy has again made this drug the target of new and attractive clinical studies, especially considering the appearance of new and less toxic platinum analogs.

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THE PHARMACEUTICAL STABILIZATION OF THE CLINICAL ANTICANCER DRUG DACH-PLATINUM-TMA (NSC271674)

P.J. Andrulis, Jr., J. Biswas, A. Troy and P. Andrulis III

I. ABSTRACT

The complex 4-carboxyphthalato-(1,2-diamino-cyclohexane)-platinum (NSC 271674; DACH-PT), has undergone limited clinical trials at Memorial Sloan-Kettering Cancer Center, New York. Heavily pretreated Phase I and Phase II patients responding to DACH-Pt therapy included those with lung, nasopharyngeal, gastric, ovarian, and bladder cancers. Renal and gastrointestinal toxicity were minimal.

Even though the complex possesses outstanding therapeutic properties, difficulties in purification and inherent instability have halted the clinical evaluation essential for its development into a commercial drug.

All purification problems have been solved; result will now be presented on attempts to stabilize the drug by varying temperature, pH, and buffer and free ligand concentration in an effort to obtain conditions under which the complex will have pharmaceutically acceptable stability while retaining superior therapeutic properties.

Results will include trade-off and sensitivity studies on buffer selection and concentration, on pH, and on temperature. Results will also be presented on the effect of molar excess as of trimellitic acid upon stability and upon in vivo antitumor activity. Development of affinity chromatography to more effectively quantitate the components of the DACH-platinum dissociation equilibrium will also be described.

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II. BACKGROUND AND APPROACH

Now that the clinical success of the complex 4-carboxyphthalato(1,2-diaminocyclohexane)platinum (DACH-Pt-TMA) has been established and we can consistantly produce it in bulk with a purity greater than 98%, stabilization of the complex under less than ideal conditions is desirable.

DACH-Pt-TMA appears highly temperature sensitive. Previous studies in other laboratories have shown breakdown of the complex in solution at room temperature after 4 hours, while it remains 90% undissociated after 24 hours in solution if stored at 4 C. Stability has also been shown to be affected by pH and buffer composition. The dissociation of the complex in aqueous solution is represented by equilibrium (1). Research has focussed on trade-off studies of temperature, pH, and buffer and trimellitic acid (TMA) concentration in an attempt to determine stabilization conditions which are optimum for pharmaceutical formulation and use. To minimize or eliminate this hydrolytic instability, varying concentrations of trimellitic acid were added to buffered solutions of the complex to force equilibrium to the left and thus stabilize undissociated complex. The optimal buffer and its concentration, pH and temperature were also determined. Stability of the complex was assayed using UV spectroscopy. However, UV profiles gave conflicting interpretations. (Figure 1) Although UV indicated substantial dissociation after several hours at room temperature, the drug was still highly active against tumors in mice. Further, TMA added to stabilize the complex interfered with the UV monitoring of the dissociation. Therefore, affinity chromatography was used in conjunction with animal screening as tools to develop alternative assays for dissociation and activity.

III. EXPERIMENTAL

Optimal buffer and TMA concentration were established by addition of DACH-Pt-TMA (98%+ purity) without excess TMA to different buffer and bicarbonate solutions and assaying solutions

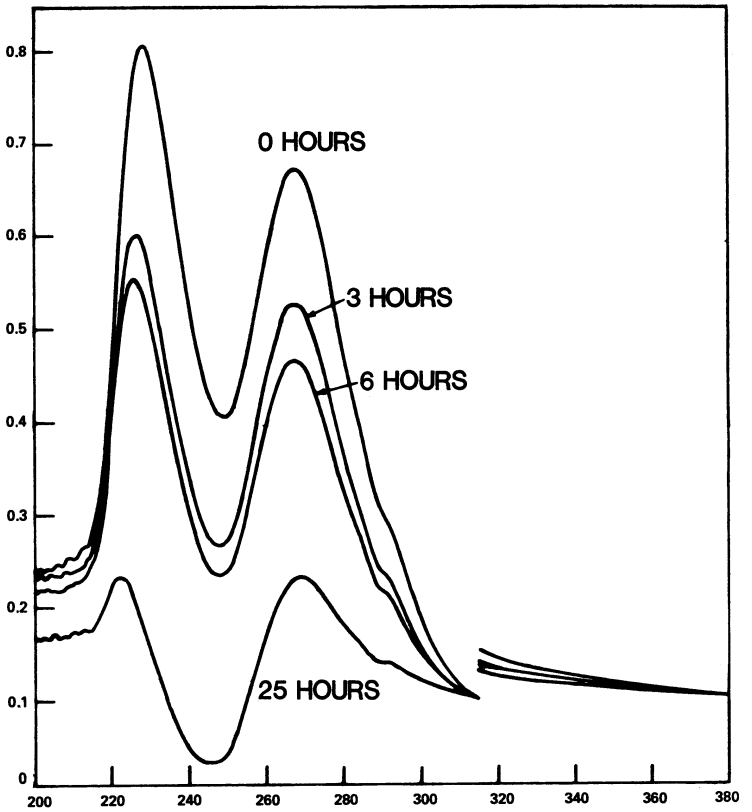

Fig. 1: Dissociation profile in UV of 4-carboxyphthalato(1,2-diaminocyclohexane)platinum (DACH-Pt-TMA) in 100 mM potassium phosphate buffer, pH 7.8, during 24 hours at room temperature.

for 2 hours at constant wave length (267nm). Varying concentrations of TMA were evaluated against the buffering capacity of the solutions to determine the maximum amount of TMA which could be added without causing adverse pH and salt concentration effects. Up to 5 molar equivalents excess TMA could be added without affecting the pH of the optimal buffer.

To assay dissociation using affinity chromatography in agarose gels, gels were chosen which would bind carboxylic acids and platinum. Iminodiacetic acid coupled to an agarose matrix with a 1,4-butanediol diglycidyl ether spacer arm was used to bind platinum (2) Diaminodipropylamine was used to bind COOH in an amide linkage using activation by a water-soluble carbodiimide (3). Zero, one or two molar excesses of TMA were added to

complex and fresh or 24 hour old solutions were loaded onto the iminodiacetic acid columns, eluted and studied by UV.

According to UV assays, less than 1 molar equivalent excess TMA did not appear to effectively inhibit dissociation. Therefore, for studies of complex used against L1210 leukemia in mice, the following stabilization protocol was devised: Freshly prepared 1% $NaHCO_3$ solutions of DACH-Pt-TMA, with 0, 1 or 2 molar equivalents of TMA, were injected into the mice containing 10^6 L1210 cells 0 hrs after preparation of solution or after allowing all solutions to stand for 24 hours at room temperature.

IV. RESULTS

A. Stability Conditions - The optimal storage conditions were 100 mM potassium phosphate buffer, pH 7.8 at which the DACH-Pt-TMA was stable for several days at 4 C while complex stored in this buffer for 24 hrs at room temperature was still active against tumors in mice.

B. Animal Data - The animal data showed results incongruous with the dissociation data seen in the UV assays, Fig. 1. Although Fig. 1 shows almost total dissociation after 24 hrs, the drug still retained anti-tumor activity.

C. Affinity Chromatography Data - At pH 7.8, the iminodiacetic acid gel was found to bind DACH-Pt-dihydrate, but not DACH-Pt-TMA, while diaminopropylamine binds TMA only. Fig. 2 shows UV spectra of column eluates from iminodiacetic acid agarose at pH 7.8, the pH at which the complex is most stable. Decomposition of complex appears to be no more than about 10-15%. Column eluates of fresh and aged DACH-Pt-TMA solutions at pH 7.8 with or without excess TMA showed 8% complex degradation with no additional TMA, 7% with one molar equivalent excess and 4% with two equivalents.

V. DISCUSSION

Previous studies on the stability of the DACH-Pt-TMA using UV spectroscopy to assay dissociation appear to be misleading. Both complexed and free TMA chromophores have virtually identical

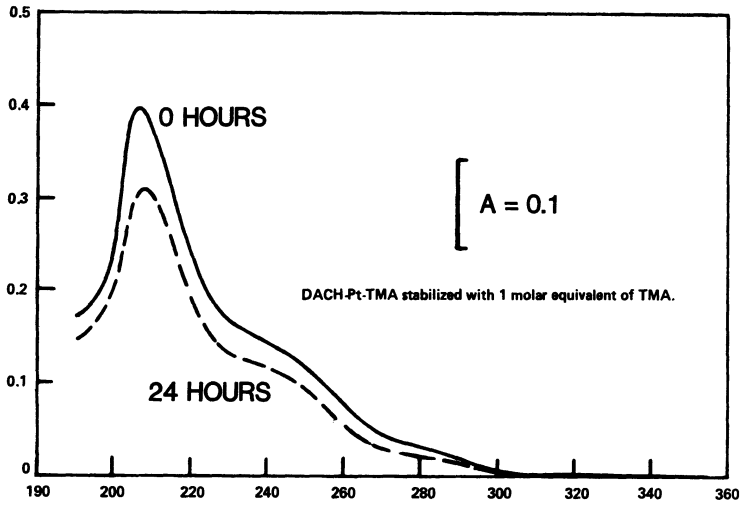

Fig. 2: UV sepctrum of DACH-Pt-TMA after elution through iminodiacetic acid agarose column of pH 7.8 at 0 hrs and 24 hrs read against a blank containing 1 molar equivalent of TMA.

UV absorbance maxima so that they are extremely difficult to distinguish. Other complexes of dissociation products which have not been identified are also forming and they may also interfere with a UV assay. Since it is currently impossible to unambiguously characterize the UV spectra, any conclusions therefrom that the complex is decomposing may be incorrect.

The animal data in Table I supports these findings: Although UV data show the complex breaking down after 24 hrs. at room temperature, in vivo data show the drug obviously retaining substantial activity. Further, various affinity column assays to date indicate little decomposition after 24 hrs, and that which does occur, from as little as 4% to no more than 15%, is similar to the decrease in the T/C ratios observed in mice treated with aged vs. fresh DACH-Pt-TMA. Under these extremely surprizing circumstances, it is not clear whether TMA is essential in inhibiting dissociation of DACH-Pt-TMA in vivo or, conversely, is totally unnecessary.

Other in vivo studies are currently underway to resolve this anomaly. Increased doses (40 mg/kg) of DACH-Pt-TMA with and without a 2 molar excess of TMA are injected after a 0 hr or a 48 hr "incubation" at room temperature. These same samples are

being subjected to iminoacetic acid affinity chromotography assays. By extending the decomposition incubation time and

Table I.

L1210 Screening of DACH-Pt-TMA

8 BDF- male mice/group
10^6 L1210 cells/mouse, IP Day 0
1 ml 25 mg/kg complex in 100 mM phosphate, pH 7.8, IP, Day 1
Controls: 7 day mean survival

Molar Excess TMA	T/C Fresh Complex	T/C Complex 24 hrs @ R.T.
0	207	169
1	200	176
2	210	200

increasing drug dosage, the ultimate effects of added TMA on dissociation will be more pronounced.

Experiments involving removing either dissociated TMA or DACH-Pt-dihydrate from the solutions of the complex using the aforementioned affinity chromatography methods will assist in determining the role of dissociation products in DACH-Pt-TMA activity.

VI. CONCLUSION

The relation between the DACH-Pt-TMA stability and its activity towards tumor cells in vivo cannot be explained simply in terms of rapid dissociation of an active complex to inactive breakdown products. Current results suggest that DACH-Pt-TMA dissociates little or much more slowly than previously reported, and that dissociated or excess TMA retards decomposition, permitting retention of in vivo activity. An alternative but potentially compatible conclusion is that longer-lived dissociated complexes are also active against tumors.

A CLINICAL STUDY OF REDUCED GLUTATHIONE AS A PROTECTIVE AGENT AGAINST CISPLATIN-INDUCED TOXICITY

S. Böhm, S. Oriana, G.B. Spatti, S. Tognella, M. Tedeschi, F. Zunino and F. Di Re

INTRODUCTION

Cisplatin is one of the most effective anticancer drugs available for the treatment of ovarian cancer, and a dose-dependent response has been documented (1,2). However, the effectiveness of high-dose cisplatin is limited by the overall (renal and nonrenal) toxicity (1). In particular, nephrotoxicity is a major dose-limiting side effect in the high-dose regimen, since at doses higher than 120 mg/m^2 there is an unacceptable incidence of renal impairment even in the presence of adequate hydration and forced diuresis (1,3). High-level hydration and hypertonic saline provide partial protection and have been included in most clinical protocols with high-dose cisplatin (1). In an attempt to improve the therapeutic index of the drug, efforts to explore methods of reducing its toxicity have continued. A variety of drug manipulations and strategies have been proposed for protecting the kidney, including protection with sulfur-containing nucleophilic compounds (4). Several thiol compounds have been shown to be effective in blocking or reducing cisplatin toxicity in experimental models (5). Among these agents, glutathione (GSH), a well-known tripeptide thiol, is of particular interest because of its excellent efficacy in animals (6), its lack of toxicity (7) and lack of interference with the antitumor activity of cisplatin (6).

The aim of this clinical study was to assess the feasibility of administration of cisplatin with reduced GSH, as a protective agent, and the role of this thiol in reducing renal toxicity.

PATIENTS AND METHODS

A total of 15 patients with histologically confirmed ovarian cancer entered the study in two phases. In a preliminary clinical experience (1984-1985), all patients (six) had measurable or evaluable advanced disease. In the second experience (1986-1987), nine patients were treated because they were at high risk of relapse after radical surgery. The characteristics of the series are summarized in Table 1. No patient had received prior chemotherapy. Evaluation parameters for toxicity and response included physical and radiographic examinations, complete blood cell count, serum electrolytes, creatinine, blood urea nitrogen, creatinine clearance and liver function tests. Standard response criteria were used.

Table 1. Characteristics of the patients

	Preliminary study (1984 - 1985)	Second study (1986-1987)
Total no. of patients	6	9
Stage of disease: I	-	6
II	1	2
III	4 (3 bulky)	1
IV	1	-
Mean age in years (range)	52 (39-70)	39 (31-48)
Karnofsky performance status	>80	>80
No. of courses with GSH	28	44

Patients received cisplatin, 90 mg/m^2 iv dissolved in 250 ml of normal saline over 30 min, and cyclophosphamide, 600 mg/m^2 iv. The standard iv hydration protocol consisted of 2000 ml of normal saline with 40 mEq KCl and 15 mEq $MgSO_4$. The treatment was repeated every 3-4 weeks. In patients with advanced disease (preliminary experience), GSH, 1500 mg/m^2 dissolved in 100 ml of normal saline, was administered iv over 15 min prior to each cisplatin injection. In the second experience, patients received the same treatment regimen with a reduced iv hydration (1000 ml fluids) and GSH, 3000 mg/m^2 dissolved in 200 ml of normal saline. Diuretics were not used. The timing of GSH administration was based on observations of timing-dependent protection in experimental

models (Sprague-Dawley rats), indicating that GSH was effective when given iv from 30 min before to 30 min after cisplatin.

RESULTS AND DISCUSSION

The patients received a total of 28 and 44 courses with GSH at 1500 and 3000 mg/m^2, respectively. GSH in this schedule and at both dose levels was well tolerated, since it did not produce appreciable side effects. In adddition, the GSH and cisplatin combination produced no unexpected toxicity. The treatment regimen had mild and transient side effects in both protocols (Table 2). As expected, the major toxic effect was nausea and vomiting, which in most patients was easily controlled with antiemetics. Hematologic toxicity was moderate and, apparently, more marked following reduction of hydration. A reduced urinary excretion of the active drugs may account for the difference in toxicity observed with the modified protocol. Anemia and thrombocytopenia were infrequent toxicities. No appreciable renal toxicity was observed. Among the patients who received reduced hydration, one course was associated with a mild and transient elevation of serum creatinine (1.6 mg/dL). No peripheral neuropathy or ototoxicity occurred with either protocol. In summary, overall our data indicate that GSH could be safely added to the cisplatin + cyclophosphamide regimen in this schedule and strongly support that GSH may provide protection against cisplatin-induced nephrotoxicity.

Table 2. Toxicity

	No. of patients	
	Preliminary study	Second study
Nausea-vomiting: grade 1	5	1
grade 2	1	6
WBC (/mm^3) 3000-4000	3	1
2000-3000	0	4
1000-2000	0	3
Platelets (/mm^3) < 100,000	0	0
Hemoglobin (g/dL) < 11	0	2
Renal toxicity (serum creatinine, mg/dL) 1.3-2.0	0	1
Neuro- or ototoxicity	0	0

The results also indicated a lack of interference of GSH on the therapeutic efficacy of cisplatin, since 6/6 patients with advanced disease, treated in our preliminary experience, achieved complete remission (pathologically documented) after a total of 40 courses (28 with GSH and 12 without GSH). Four patients achieved complete pathologic remission after five courses of therapy with GSH. All patients are alive and five of these have remained disease free after more than 2 years of follow-up. Only one patient relapsed after a 20-month progression-free interval. In the series of nine patients treated with adjuvant therapy, seven had no evidence of disease after five courses, and two are still under treatment.

With a limited number of patients in the feasibility study, no accurate assessment of the relative therapeutic value of this regimen including GSH can be given. The therapeutic results of the preliminary study support previous observations in experimental systems showing that the therapeutic efficacy of cisplatin is not impaired by GSH (6). Since GSH does not reduce the antitumor activity of cisplatin, the use of GSH may be an alternative safer method for delivering high doses of cisplatin.

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IMMUNITY IN PATIENTS TREATED WITH *CIS*-PLATINUM

G. Cartei, M. Comelli, E. Contessi, C. Toso and M. Bramezza

INTRODUCTION

Cis-platinum (DDP) is one of the most active compounds available in the treatment of cancer. Effects of DDP on the immune system have recently been explored (1, 2, 3). Initial evidence appeared to classify DDP among immunosuppressive agents: graft-versus-host responses were suppressed in animals treated with this drug (4); moreover the growth of B and T cells in colture (5) was inhibited by DDP, as well as monocyte chemotaxis (6).

In addition to immunosuppressive actions, recent works suggest that DDP may also function to stimulate some immune processes (7). It can stimulate spontaneous monocyte-mediated cytotoxicity (8), an in vitro measure of the ability of human peripheral blood monocytes to lyse ^{51}Cr-labelled red cell targets. Antibodies have also been elicited in mice treated with cis-platinum (9). The enhancement of naturally occurring immune function is another possible mechanism by which DDP, as well as other antineoplastic agents, produces tumor regression in vivo.

Aim of our study is to explore the immunological effects due to DDP given in monochemotherapy in a group of lung cancer patients, by measuring lymphocyte subpopulations and variations of serum levels of immunoglobulins.

MATERIALS AND METHODS

Nine patients, 8 males and 1 female, aged from 51 to 69 (median 57), all with measurable lung cancer, have been treated with DDP alone (Table 1).

Table 1. Patients treated with cis-platinum.

n.	M/F	age	TNM	hist.	pretreat.	n. of cycles	cumulative DDP dose	DDP effectiveness
1	M	55	M1-renal	A	-	2	240 mg/m^2	P
2	M	51	M1-pleura	A	MMC (1 month before)	3	25 mg in pleura days 1, 4 /cycle	NC
3	M	69	M1-bone	A	MMC (2 months before)	4	480 mg/m^2	NC
4	F	56	M1-pleura	A	-	3	360 mg/m^2	NC
5	M	57	M1-bone	S	antalgic RT	1	120 mg/m^2	P
6	M	63	M1-bone and cutis	S	-	2	240 mg/m^2	P
7	M	58	T3NXM0	S	-	6	720 mg/m^2	NC
8	M	57	M1-bone	LC	-	5	600 mg/m^2	NC
9	M	59	T3N2M0	LC	-	2	240 mg/m^2	NC

A=adenocarcinoma, S=squamous ca, LC=large cells ca; MMC=mitomycin (10 mg/m^2 every 3 weeks; 3 cycles in each patient), RT=radiotherapy; P=progression, NC=no change.

Dose of DDP (except in patient n. 2) was 40 mg/m^2, days 1-2-3 every three weeks. Therapy was delayed if WBC < 4000/mm^3 and/or platelets < 100000/mm^3; cycles were from 1 to 6 (median 3) with a median cumulative DDP dose of 300 mg/m^2 (range 120 to 720 mg/m^2). Therapy was delayed because of thrombocytopenia in patient n. 1 (after the 1st cycle) and leukopenia in patient n. 3 (after the 1st and the 3rd cycle), n. 7 (after the 2nd one) and mainly in n. 9 (after each one).

DDP was always given in the morning, over 60 min in 250 ml normal saline, with one-hour prehydratation by 250 ml normal saline with

20 mEq KCl, furosemide 40 mg and by means of one-hour posthydratation by 10 % mannitol 500 ml. Alizapride (N-(1-allyl-2-pyrrolidylmethyl)-2-methoxy-4,5-azimido benzamine)(Limican) 400 mg in 125 ml saline, was used as antihemetic against DDP effects, as given before prehydration. Therapy was stopped as progression of disease was clearly demonstrated.

The study included various tests, to be performed just before each therapy cycle (Table 2). Blood sampling was always made between 8 and 9 a.m. To obtain a more correct ratio between helper and suppressor T cells, fluorescence microscope, instead of flow-cytometric analysis (10), was used.

Statistics was made by means of t Student's test for paired data, each patient being control of himself.

Table 2. Available tests.

- full blood counts and differential leukocytes counts	COULTER COUNTER AND HAEMALOG
- lymphocyte subpopulations: total B, total T, T helper, T suppressor	INDIRECT IMMUNOFLUORESCENCE BY "ORTHO" M-Ab
- total IgE	RIA TEST (PHARMACIA)
- IgA, IgG, IgM, C3, C4	NEPHELOMETRY AFTER TREATMENT WITH BECKMAN ANTISERA

RESULTS

WBC were clearly affected by DDP chemotherapy: there was an important decrease in their number expecially after three cycles, with a 59.1 % fall ($p < 0.025$).

The same happened for neutrophils with a 69.1 % fall ($p < 0.025$) after three cycles, while lymphocytes did not present significative variations.

Monocytes and eosinophils showed a decrease after two DDP cycles,

respectively of 17.9 % ($p < 0.01$) and 56.6 % ($p < 0.005$), but while the first ones presented a tendency to recover, the second ones remained at low values (Table 3).

Table 3. WBC and differential count during DDP therapy ($\bar{x} \pm 2SD$).

	before the 1st	2nd	3rd	4th cycle
WBC (n/mm^3)	10443±2215	8843±4104°	8314±5150*	4266±208*
Neutrophils (n/mm^3)	7187±3196	6288±3532°	4288±1934**	2220±221*
Lymphocytes "	1841±437	1919±718°	1697±456°	1681±172°
Monocytes "	428±181	579±213°	351±190**	242±147°
Eosinophils "	212±99	169±128°	92±101•	96±58*
(n. of patients)	(9)	(7)	(7)	(3)

° NS, * $p < 0.025$, ** $p < 0.01$, • $p < 0.005$

Lymphocytes subpopulations (OKB2, OKT3, OKT4, OKT8) did not present statistically significative variations, following the general trend of lymphocytes. The various subpopulations maintained their initial balance (Table 4).

Table 4. Lymphocytes subpopulations during DDP therapy ($\bar{x} \pm 2SD$).

	before the	1st	2nd	3rd	4th cycle
OKB2 (%)		6.4±4.2	5.5±1.3°	11.5±6.4°	6.0±2.6°
" (n/mm^3)		103±49	160±101°	249±217°	103±52°
OKT3 (%)		64±11	63±9°	75±15°	69±2°
" (n/mm^3)		1152±474	1145±454°	1527±905°	1165±102°
OKT4 help. (%)		42±7	41±3°	54±26°	47±8°
" "(n/mm^3)		748±240	758±340°	1158±962°	805±207°
OKT8 suppr. (%)		22±10	22±7°	24±10°	23±9°
" " (n/mm^3)		341±222	391±148°	432±60°	377±111°
OKT4/OKT8		2.0±1.0	1.9±0.5°	2.6±2.2°	2.3±1.1°

° NS

IgA and IgG serum concentration had the same behaviour: after the first cycle of chemotherapy they presented a significative increase, consistent in each patient, respectively of 2.3 % ($p < 0.05$) and 4.3 % ($p < 0.01$). After this, both IgA and IgG had a nadir before the third cycle and subsequently a return to the basal situation (Fig. 1 and Fig. 2).

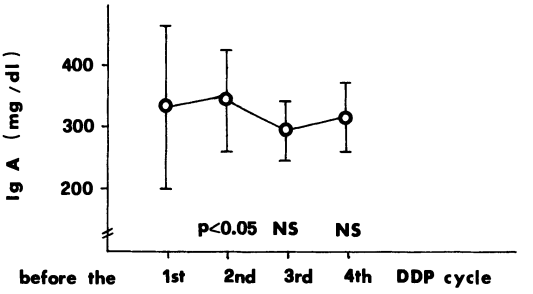

Fig. 1

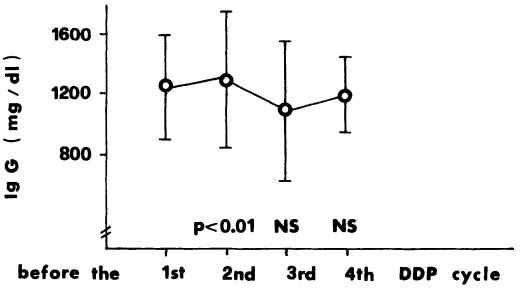

Fig. 2

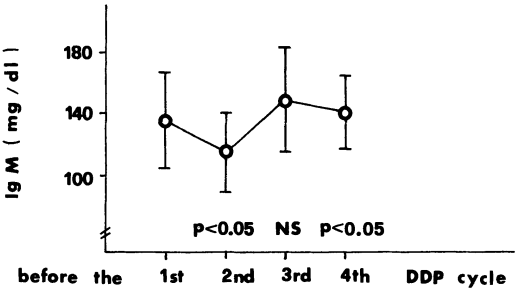

Fig. 3

IgM showed a different trend: after the first cycle, there was a 14 % nadir ($p < 0.05$) and during the following cycles a tendence to recover to the initial value (Fig. 3).

The number of studied subjects is still not large and the study is open to further accrual of patients; however cis-platinum has shown no effect on the T helper/T suppressor ratio and no relevant or appreciable modification on serum immunoglobulins; B lymphocytes were also unaffected.

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EARLY AND PROGRESSIVE GLOMERULAR KIDNEY TOXICITY DURING *CIS*-PLATINUM THERAPY

G. Cartei, E. Contessi, M. Comelli, O. Geatti and E. Cattaruzzi

INTRODUCTION

Cis-diammine-dichloroplatinum (DDP) largely used in cancer therapy has major and reversible toxicities like nausea, vomiting (1) and kidney tubular disfunction. Hyperhydratation, furosemide, KCl supplements are effective in circumventing the tubular nephrotoxic damage exerted by the drug (2, 3, 4).

Preliminary prospective observation by our group indicated also a progressive and cumulative glomerular toxicity, as assessed by Technetium-99 diethylenetriaminepentacetic acid (99Tc-DTPA) studies (5, 6). Subsequently, Fjeldborg et al. (7) in a retrospective analysis demonstrated a 12.5 % fall in glomerular filtration rate of 51Cr-EDTA clearance, 16 to 52 months after polychemotherapy including DDP. These authors had no information on the time of beginning of the DDP toxicity.

MATERIALS AND METHODS

Thirty-two patients, 19 males and 13 females, aged from 42 to 66 years (median 57, mean 56) have been treated with DDP-based chemotherapy regimens. DDP alone was administered to 4 patients; other various combinations are given in Table 2. Median cumulative DDP dose was 270 mg/m^2 ranging from 100 to 720, with a median number of administered DDP cycles of 3 (from 1 to 6)(Table 1).

Table 1. Data from 32 patients with cancer treated with DDP.

n	M/F	age	diagnosis site, TNM	DDP cumulative dose (mg/m^2)	chemotherapy	n. cycles
1	M	47	lung T3 N2 M0	720	DDP alone	6
2	M	58	" T3 NX M0	360	" "	3
3	M	57	" M1-lymph.	480	" "	4
4	M	58	" M1-pleura	360	" "	3
5	M	54	" M1-lymph.	600	DDP, ADM	5
6	M	61	" T2 N2 M0	240	" "	2
7	M	62	bladder M1-lymph.	100	DDP, 5FU	1
8	M	57	rinopharynx N3	180	" "	2
9	M	66	lung M1-bone	360	DDP, DHAD	3
10	M	53	" M1-bone	240	" "	2
11	M	51	" T3 N1 M0	480	DDP, VP16	4
12	M	51	" T3 NX M0	160	DDP, VLB	4
13	M	60	" T2 N2 M0	480	DDP, 5FU, CTX	4
14	M	63	" T3 N2 M0	360	" " "	3
15	M	53	" T3 N2 M0	600	" " "	5
16	M	57	" T3 N2 M0	480	" " "	4
17	M	48	gastric M1-pleura	270	" " "	3
18	F	56	breast M1-bone, cutis	270	DDP, ADM, CTX	3
19	F	56	" T4b N1 M0	180	" " "	2
20	F	65	" M1-bone	270	" " "	3
21	F	56	" M1-liver	270	" " "	3
22	F	61	" M1-bone	450	" " "	5
23	F	56	" M1-multiple	360	" " "	4
24	F	57	" M1-bone	270	" " "	3
25	F	44	" M1-bone	270	" " "	3
26	F	45	" M1-bone	450	" " "	5
27	F	57	" M1-multiple	180	" " "	2
28	F	64	" T4 N2 M0	180	" " "	2
29	F	54	" M1-cutis	270	" " "	3
30	F	60	" M1-abdomen	270	" " "	3
31	M	48	testis N2	300	DDP, BLM, VLB	3
32	M	51	oral T3 N1 M0	150	DDP, BLM, MTX	3

(CTX=cyclophosphamide, ADM=adriamycin, DHAD=mitoxantrone, 5FU=5-fluorouracil, VLB=vinblastine, VP16=etoposide, BLM=bleomycin, MTX=methotrexate)

Dose schedule of DDP and other drugs are given in Table 2.

Table 2. DDP combinations: each regimen was repeated every 21 days.

Regimen	Dosage ($mg/m^2/day$)	Days of administration
DDP	40	1, 2, 3
DDP	40	1, 2, 3
ADM	30	1, 2, 3
DDP	40	1, 2, 3
DHAD	5	1, 2, 3
DDP	100	6
5FU	800	1, 2, 3, 4, 5
DDP	40	1, 2, 3
VP16	120	1, 2, 3
DDP	40	6
VLB	1.5	1, 2, 3, 4, 5
DDP	40	1, 2, 3
5FU	400	1, 2, 3
CTX	400	1, 2, 3
DDP	30	1, 3, 5
ADM	40	1
CTX	400	1, 3, 5
DDP	20	1, 2, 3, 4, 5
BLM	18	2, 9, 16
VLB	6	1, 2
DDP	50	4
BLM	8	1
MTX	40	1

(abbreviations like in Table 1)

DDP was always given over 60 minutes as diluted in 250 ml normal saline, with one-hour-prehydratation by 250 ml normal saline with 20 mEq KCl, furosemide 40 mg and by means of one-hour-posthydratation by 10 % mannitol 500 ml. Alizapride (N -(1-allyl-2-pyrrolidylmethyl) -2-methoxy-4,5-azimido benzamine)(Limican) 400 mg in 125 ml saline,

before and after DDP, was used as effective antihemetic. Patients were not given allopurinol, but some of them received both oral and parenteral Ca and Mg during DDP-chemotherapy, because of clinical symptoms.

Evaluation of disease response to antiblastic chemotherapy was made just before therapy, and subsequently before the 3rd, 5th and 7th cycle. Chemotherapy was discontinued if complete, partial or at least minimal remission were not attained.

The study prospectively included various tests (Table 3) to be done just before each therapy cycle.

Statistics was made by means of t Student's test for paired data, each patient being control of himself.

Table 3. Blood and urine tests available in present study (before each therapy cycle).

- Hb, MCHC, PCV, RBC, WBC and differential count, platelets, Fe
- urea, uric acid, creatinine
- urinalysis, endogenous creatinine clearance (24 h urine)
- Addis test (eritrocytes number/24 hours)
- 99m Tc-DTPA imaging renogram and kinetics: time of peak (8), mean transit time (9), retention time (10), elimination time (10)

RESULTS

Most of the patients maintained normal serum creatinine during DDP therapy. Only 3 patients (9.3 %) out of the 32 had a serum creatinine increase above 1.2 mg/dl. In patient n.8 (see Table 1) creatinine rised from 1.0 to 1.7 mg/dl before the second cycle of DDP (after 75 mg; 40 mg/m^2), but was normal before the following other four cycles. In patient n.20 serum creatinine rised from 1.0 to 2.8 mg/dl before the third cycle (DDP total dose 270 mg, i.e. 180 mg/m^2). The DDP cycle was postponed and serum creatinine spontaneously recovered to normal value within seven days; no creatinine increase over 1.2 mg/dl was observed after the following cycle. The third patient

(n.29), 21 days after the 5th cycle (cumulative dose: 1000 mg, i.e. 500 mg/m^2) showed a serum creatinine of 2.3 mg/dl (BUN: 42 mg/dl). Further therapy was refused by the patient and serum creatinine spontaneously recovered to normal levels.

In the entire group, levels of BUN and uric acid in the absence of allopurinol therapy tended not significantly to decrease with a nadir just before the third cycle.

The 99m Tc-DTPA kinetics revealed a significative delay in the peak time (from 5.43 to 7.28 min; increase by 34.6 %; $p < 0.05$) and in the mean transit time (from 3.59 to 4.21 min; increase by 17.3 %; $p < 0.05$). Mean values already exceeded the normal values just after 1-2 cycles of DDP. Retention time showed small, not significative increase (8.8 %)(Table 4).

Table 4. 99m Tc-DTPA study ($\bar{x} \pm 2SD$).

	before the 1st	2nd	3rd	4th	5th cycle
TP (<5 min)	5.43±2.26	6.05±3.12*	6.62±2.85*	6.85±3.50*	7.28±3.51*
MTT (<3.5 min)	3.59±1.11	3.58±1.08°	4.12±1.20°	4.18±1.98*	4.21±0.71*
RT (<1.5 min)	1.47±0.63	1.79±0.67°	1.62±0.66°	1.74±1.84°	1.60±0.41°
ET (<5 min)	3.81±1.34	4.00±1.59°	4.50±1.59*	4.68±2.30°	5.10±1.85°

(TP=time of peak, MTT=mean transit time, RT=retention time, ET=elimination time; ° NS, * $p < 0.05$)

Haematocrit (PCV) and haemoglobin (Hb) concentration reduced progressively during DDP therapy. PCV fall was already significative after the first cycle; PCV nadir happened before the 4th cycle (% difference = -20.3). The same was for Hb concentration.

The Addis test revealed a 24 hrs urinary loss of eritrocytes (x 10^3): from basal 688±627 to 358±254 after 2 cycles and to 415±571 after 3 cycles, without significance. Subsequently it rised to pre-treatment levels.

Renal toxicity due to DDP is usually represented by an acute tubular disfunction (11, 12, 13), which has been ascribed to the high renal cortical DDP concentration (14). The tubular toxicity is considered completely recovered within 3 weeks and moderate or high doses of DDP are consequently administered every 21 days.

Incidence of nephrotoxicity is not higher in patients receiving higher cumulative doses of DDP and increases in serum creatinine levels may occur sometimes after discontinuation of the drug (15, 16).

Our previous studies have revealed that also glomerular function could be affected by DDP administration (5, 6). As furtherly the study progressed to present results, a statistical glomerular function depression was confirmed. In the 32 consecutive patients studied, an early and progressive delay in 99m Tc-DTPA time of peak and mean transit time (both used for assessing the glomerular filtration rate) was observed. Reduction of glomerular flux and filtrate was not accompained by increase in blood urea, creatinine or other tests; to the contrary we observed a progressive reduction of BUN and uric acid (no patient received allopurinol). Blood pressure never tended to rise but sometimes a drug (midodrine 7.5 mg/day)was requested to rise a lowered blood pressure.

In conclusion, administration of DDP both alone either in combination chemotherapy is accompained by glomerular and tubular kidney toxicity. Present results showed that glomerular damage starts early (already after the first DDP cycle), is progressive and cumulative and also long-lasting after stopping the DDP therapy (7).

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HEPATIC ARTERIAL INFUSION OF CISPLATIN AS A SINGLE AGENT OR IN COMBINATION WITH VINBLASTINE FOR BREAST CANCER METASTATIC TO THE LIVER

G. Fraschini, G. Fleishman, H. Carrasco, C. Charnsangavej and G. Hortobagyi

INTRODUCTION

Breast cancer patients with hepatic metastases that are refractory to front-line chemotherapy respond poorly to additional systemic therapy. In a study of more than 100 patients treated with systemic continuous infusion vinblastine (V) we observed a 37% overall response rate, but responses were documented in only 13% of the hepatic metastases (1). Regional delivery may allow to increase the tumor exposure to suitable antitumor agents. The reported trials of intra-arterial regional chemotherapy have generally shown some advantage for this delivery route (2).

As part of our effort to assess the antitumor activity and toxicity of hepatic arterial infusion (HAI) chemotherapy in breast cancer patients whose disease was predominantly confined to the liver, we have evaluated the therapeutic efficacy of cisplatin (CDDP) in two sequential trials, as a single agent initially, and in combination with V subsequently. CDDP has shown significant activity in untreated breast cancer patients (3), although its activity in pretreated patients is modest (4 - 6). This agent has dose-dependant activity that may be enhanced by regional delivery (7 - 8). V, given as a 5 day continuous infusion, has significant activity in pretreated breast cancer patients that appears dose-dependant (1). This agent is partly metabolized by the liver. Theoretically, this property would allow to use larger doses when HAI delivery is used, as compared to systemic delivery, without increasing the systemic toxicity. Additionally, CDDP and V have incompletely overlapping toxicity, which would allow to use them in combination at doses

approaching the standard single-agent doses.

PATIENTS AND METHODS

Breast cancer patients with liver metastases and refractory to one or more chemotherapeutic regimens were included in the two treatments. Patients with limited extra-hepatic metastases of minor prognostic significance were also included. Additional eligibility requirements comprised adequate renal and bone marrow functions, a Zubrod performance ≤ 3, and a signed informed consent approved by the Institutional Review Board. For each treatment the patients were admitted to the hospital. New percutaneous catheters were inserted into the femoral, axillary, or high brachial artery by the Seldinger technique (9) and positioned within the proper or common hepatic artery. Volumetric infusion pumps were employed to maintain a steady flow rate of the chemotherapy solution and the standard hydration regimen with mannitol diuresis was administered with CDDP. The following treatment schedules were observed:

Trial I - CDDP 120 mg/m^2 and 3,000 units of sodium heparin were dissolved in 500 - 1,000 ml of normal saline solution and infused over 2 to 4 hrs.

Trial II - CDDP 100 mg/m^2 was administered as in trial I and followed by a daily continuous infusion of V 1.7 mg/m^2 for 5 consecutive days. Each V dose was diluted in 1,000 ml of 5% dextrose solution with 15,000 units of sodium heparin and infused over 24 hrs. The heparin dose was titrated to maintain a partial thromboplastin time of about 1½ times the upper normal value.

The treatments were generally repeated at 4 week intervals and the chemotherapy doses were increased or decreased by 20% in subsequent courses, based on the degree of myelosuppression or other toxic effects.

The evaluation of response was done by imaging techniques including computerized tomography, hepatic angiography, and sonography. When applicable, responses by imaging techniques were correlated with physical findings and serum carcinoembryonic antigen determinations. Response was assessed in accordance with WHO criteria (10).

RESULTS

Trial I - Twenty-six patients were evaluable. The pretreatment characteristic included: median age of 47 (range, 28 - 66), median Zubrod performance score of 1 (0 - 3) and median number of previous chemotherapeutic regimens of 2 (1 - 7) with a median duration of 23 mos. (3 - 49). Seven patients had extra-hepatic metastases.

The responses seen are shown in Table 1. The 95% confidence interval for the response rate observed was 7% - 38%. None of the responding patients had extra-hepatic metastases. The responding patients had a median time to progression (TTP) of 15+ wks (range, 8+ - 55) and a median survival (S) of 11 mos. (range, 8 - 22).

Trial II - The pretreatment characteristics of 33 evaluable patients included: median age of 42 yrs. (range, 29 - 75), median Zubrod performance score of 1 (0 - 3), median number of previous chemotherapeutic regimens of 2 (1 - 6) with a median duration of 14 mos (4 - 59). Sixteen patients had extra-hepatic metastases.

The results obtained are shown in Table 1. The 95% confidence interval for the response rate that we observed was 18 - 52%. For responding patients the median TTP was 31 wks (range, 16+ - 74) and median S was 11 mos (range, 5 - 19). Three responding patients had a significant improvement of their performance score and one achieved a partial remission of chest wall metastases as well. Of 14 patients that were considered as treatment failures, eleven received only one course of the regimen, eight because of objective or subjective treatment intolerance, one because of hepatic arterial occlusion, and two that died of rapidly progressive disease within two weeks of the onset of therapy. Hepatic arterial occlusion also prevented further therapy in a patient who achieved a minor response after the first course. Of 21 patients who were able to complete at least two courses of the regimen, eleven (52%) achieved a partial remission and seven had a minor response.

TOXIC EFFECTS

The adverse effects that occurred in the two treatment trials are shown in Table 2. Single-agent CDDP was generally tolerated at the protocol dosage. Nausea and emesis were the major adverse effects.

The toxicity of the trial II regimen was significant. It often demanded reduction of the doses and was a major determinant of treatment failure, especially in elderly patients and in those with poor performance. Myelosuppression was severe and associated with major infections in three patients; fatigue and paralytic ileus were intolerable to some. Inappropriate secretion of antidiuretic hormone occurred in one patient who erroneously received a V daily dose of 2 mg/m^2. One patient developed a catheter-induced thrombosis of the external iliac and common femoral arteries that resisted to fibrinolytic therapy, and resulted in a limb amputation. Complete occlusion of the hepatic arterial circulation was the result of diffuse arteritis that was attributed to the known sclerosing property of V. This complication prevented from administering further treatments to the patients affected, who received the same combination chemotherapy intravenously at reduced doses. We found no correlation of arterial occlusion with response.

DISCUSSION

These trials indicate that both CDDP regimens may be of benefit in individual breast cancer patients with hepatic metastases who have become refractory to systemic chemotherapy. If we consider that systemic cisplatin has reportedly induced remissions in pretreated patients with soft tissue or lung metastases only (5 - 6), our results suggest that its activity is increased by regional delivery.

The pretreatment characteristics of the patients included in these two trials were quite similar, but the regimens were investigated in sequential rather than randomized populations and their results are not directly comparable. Yet, the activity of the CDDP-V regimen appears superior to that of CDDP alone and it compares favorably with the activity shown by these agents when they were administered intravenously (1, 4 - 6). Almost all of the patients who received at least two courses of the combined HAI regimen obtained objective remissions or minor improvements. Unfortunately, the patient tolerance of this regimen was significantly limited by the adverse effects and this diminished its therapeutic value.

The activity of HAI of CDDP should be further assessed in conjunction with new, improved supportive measures that may limit its

toxicity, with proper patient selection, and in combination with other antineoplastic agents suitable for HAI.

Table 1. Response by Treatment Regimen

Response	Trial I (CDDP)	Trial II (CDDP + V)
Patients Evaluable	26	33
Partial Response	5 (19%)	11 (33%)
Minor Response	3	8
No Response	18	14

Table 2. Adverse Effects by Treatment Regimen

Type		% Of Patients Trial I (CDDP)	% Of Patients Trial II (CDDP + V)
Catheter-related			
Hepatic arterial aneurysm		14	3
Arterial occlusion at insertion site		--	3
Drug-related			
Nausea/emesis		97	100
Fatigue, malaise		28	15
Paralytic ileus		--	9
Fever with leukopenia		21	18
Sepsis		--	6
Death from septic complications		--	3
Mild nephrotoxicity		10	12
Mild ototoxicity		7	---
Inappropriate ADH secretion		--	3
Hepatic arterial occlusion		--	30
Myelosuppression			
Granulocyte nadir ($cells/mm^3$)	Median	1,700	450
	Range	200 - 4,300	0 - 2,200
Platelet nadir ($x\ 10^3\ cells/mm^3$)	Median	105	76
	Range	14 - 212	5 - 363

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COMPARISON BETWEEN A HUMAN SMALL CELL LUNG CARCINOMA CELL LINE (GLC_4) AND AN ADRIAMYCIN (GLC_4-ADR) AND A CDDP (GLC_4-CDDP) RESISTANT SUBLINE. A PRELIMINARY REPORT

G.A.P. Hospers, N.H. Mulder, B. De Jong, J.G. Zijlstra and E.G.E. De Vries

INTRODUCTION

Many relatively effective strategies for treatment of nearly all forms of chemosensitive cancer include combinations or alternations of agents that are supposed to have different modes of actions. In part these strategies are based on the assumption that such differing modes of action will ensure that tumor cells that are not sensitive to one agent will react to the other. This assumption requires that a close relation exists between mode of action of a drug and the mechanism of resistance operating for that drug in tumor cells. We have studied this hypothesis for two drugs that are considered to be active cytotoxic agents in a number of human malignancies, including small cell lung cancer, namely Adriamycin (ADR) and CDDP. This preliminary report concerns some details on the pheno- and genotype, and resistance mechanisms of a platinum (GLC_4-CDDP) and of an ADR resistant (GLC_4-ADR) and a CDDP resistant (GLC_4-CDDP) cell line, both derived from a sensitive human small cell lung cancer cell line (GLC_4).

MATERIALS AND METHODS

Cell lines.

The GLC_4 cell line is derived from a pleural effusion of a patient with small cell lung cancer. The GLC_4-ADR subline is developed by continuous exposure to ADR. The GLC_4-CDDP subline is established by continuous exposure to CDDP for 12 months and thereafter intermittent exposure for 3 months.

Clonogenic assay (CA).

For cross-resistance determination one 1 hr drug incubations with ADR, CDDP and melphalan are performed. After washing 3 times with phosphate buffered saline the cells are plated. The CA is performed as described earlier (1). For GLC_4 3000 cells and for GLC_4-ADR and CDDP 5000 cells are plated per dish. The resistance factor (RF) is expressed as the ratio between the drug concentrations leading to a 50% decrease in colony growth in the resistant and the sensitive cell line.

Karyotyping.

Chromosome slides are prepared according to standard cytogenetic techniques.

Intracellular drug levels.

For intracellular ADR, ADR is determined according to Bachur et al (2) by measurement of total fluorescence of the supernatant of 5 x 10^5 GLC_4 or GLC_4-ADR cells extracted with 0.3 N HCl/50% ethanol on the Kontron spectrofluorometer (excitation 474 nm and emission 549 mm. The cells are exposed to the drug for 1 hr, thereafter cooled to 0^oC and washed 3 times.

For intracellular CDDP, platinum is determined with a model 1275 atomic absorption spectrophotometer with a model GMA-95 graphite tube atomizer and autosampler (Varian Techtron). A wavelength of 265.9 nm with a lamp current of 10 mA and a spectral bandwidth of 0.5 nm. For calibration platinumchloride solution in HNO_3 (65%) is used. GLC_4 or GLC_4-CDDP cells (2 x 10^7) are incubated with CDDP for 1 hr, washed with PBS, dried and the pellet is resuspended in HNO_3 (65%).

Sulfhydryl compounds and related enzymes.

In the logarithmic phase cells of the 3 cell lines were harvested for glutathione (GSH), acid-soluble sulfhydryl compounds (AS-SH), glutathione S-transferase (GST), and glutathione reductase as described previously (3).

For protein determination the Lowry assay was used (4).

DNA damage.

For ADR damage, strand breaks were measured by alkaline unwinding and determination of ethidium bromide (5) fluorescence. GLC_4 and GLC_4-ADR cells were incubated for 1 hr.

For CDDP, cross-links were measured with the ethidium bromide fluorescence cross-link assay for whole cells (6). GLC_4 and GLC_4-CDDP cells are incubated for 1 hr.

RESULTS

Growth characteristics.

All 3 cell lines have a doubling time of 24 hr. The GLC_4 and GLC_4-ADR lines grow partly floating, partly attached, the GLC_4-CDDP line grows mainly attached. Cloning efficiency is 33% for GLC_4, 15% for GLC_4-ADR and 17% for GLC_4-CDDP. There are no morphological differences with light-microscopy.

Cross-resistance.

The RF of GLC_4-ADR with GLC_4 is 40 for ADR, 2.1 for CDDP, 0.4 for melphalan. The RF of GLC_4-CDDP with GLC_4 is 4.0 for ADR, 8.0 for CDDP, 5.0 for melphalan.

Karyotype.

All 3 lines are aneuploid with many structural abnormalities. There is no special abnormality related to one of the cell lines. The number of double minutes is significantly higher in the GLC_4-ADR cell line than in the GLC_4 line and significantly lower in the GLC_4-CDDP line than in the GLC_4 line.

Intracellular drug levels.

There is a 40% lower ADR concentration in GLC_4-ADR cells compared with GLC_4 cells at 10 and 20 μM ADR 1 hr drug incubation. For the GLC_4-CDDP line a 21% lower platinum content was found in the range from 10-50 μg/ml CDDP 1 hr drug incubation compared to the GLC_4 cell line.

Sulfhydryl compounds and related enzymes.

GSH in the GLC_4-ADR cell line is significantly reduced (59%) and in the GLC_4-CDDP cell line 2.5x increased compared to GLC_4. AS-SH is significantly reduced in the GLC_4-ADR line and 1.3x elevated in the CDDP resistant line. GST is identical for the 3 lines. GR is 15x elevated in the GLC_4-ADR line and identical in the GLC_4-CDDP line.

Protein content cells.

The protein content is identical in GLC_4 and GLC_4-ADR cells

(2.0 mg/10^7 cells) and reduced in the GLC_4-CDDP cells (1.4 mg/10^7 cells).

DNA damage.

The amount of strand breaks induced by ADR is lower in the ADR resistant line than in the GLC_4 cell line, even at identical intracellular ADR levels.

In GLC_4-CDDP cells CDDP incubation results in 45% reduction of interstrand cross-links compared to GLC_4 cells.

DISCUSSION

GLC_4-ADR and GLC_4-CDDP are quite different cell lines as they vary in a spectrum of characteristics. The gross morphological appearance is different. As a result GLC_4-CDDP is in that respect no longer characteristic of the variant subtype form of SCLC as defined by Carney (7). Cellular protein content is reduced in the CDDP resistant line and GSH and AS-SH is raised only in this line. Finally, if the presence of double minute chromosomes is considered to reflect genetic stability, the GLC_4-CDDP line must be considered to be much more stable than the ADR resistant counterpart or even than the sensitive parent cell line.

The drugs to which the cells were exposed are considered to have no cross reactivity. ADR is supposed to act by interfering with topoisomerase 2, leading to lethal breaks in the DNA strings. Resistance is thought to be the effect of an outward directed efflux of the drug leading to lower intracellular concentrations. CDDP is thought to produce cross-links in the DNA, and resistance mechanisms have less clearly been defined.

Nevertheless, some similarities are present as far as the resistance characteristics for the two different drugs are concerned in these differing cell lines that have a common parentage.

The most important common feature is the presence of cross resistance in the CA, be it in a low degree. However, resistance with a factor 2 could if present in a patient be enough for the difference between a complete remission and no response. Also to some degree the location of the relevant mechanisms may be the

same. In both cell lines an important aspect is the lower cellular drug concentration. We do not yet know the nature of the drug barrier, but it is quite well possible that in both situations this will be found to be located at the membrane level. In both cell lines an important mechanism is also operating at the nuclear level, influencing the type of DNA damage that occurs or remains in the resistant cells. As yet it is not certain if these mechanisms, that probably involve DNA repair, have aspects in common.

We conclude from the experiments described here, that without specific studies on the type of resistance present, it is impossible to state that drugs or combination of drugs will not be cross resistant in the clinic.

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THE EFFECT OF DIETHYLDITHIOCARBAMATE (DDTC) ON RESPONSE, TOXICITY AND PHARMACOLOGY OF CISPLATIN IN PATIENTS WITH RECURRENT SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

A. Kramer, J. Paredes, I. Dimery, K. McCarthy, T. Felder, R. Newman and W.K. Hong

Squamous cell carcinoma of the head and neck (SCCHN) accounts for approximately 5% of the cases of cancer in the United States. It is estimated that in 1986, 40,400 new cases of cancer occurred with 13,000 deaths (1). A local recurrence rate of up to 60% with a distant metastatic rate of 30% has been seen with patients presenting with Stage III and IV SCCHN treated with surgery and/or radiotherapy (2-4). Most patients whose disease recurs will die of their head and neck cancers.

Chemotherapy for recurrent SCCHN has been utilized for the past two decades. Initially, single agent trials utilizing Methotrexate, Bleomycin, and Cisplatin were conducted, with response rates ranging between 20 to 30% and with duration of responses lasting 2 to 4 months (5). Combination chemotherapy trials utilizing several active agents in SCCHN have yielded response rates of 30-65%, but no survival benefit has been demonstrated (2,6-8). In 1984 Kish reported the results utilizing Cisplatin (100 mg/m^2 Day 1) with a 4-day infusion of 5-Fluorouracil (1000 mg/m^2 D1-4) in patients with recurrent SCCHN (9). The overall response rate of 70% with a complete response rate of 27% was very encouraging. Since that time other investigators have reported lower response rates, in the range of 11 to 62% (10,11), using this same regimen.

It is well known that the dose-limiting side effects of Cisplatin have been renal and neurologic toxicities. Investigators using the CisDDP/5-FU regimen recognize the difficulty of administering more than 4 to 5 courses of this regimen because

of its cumulative toxicities.

Diethyldithiocarbamate (DDTC), a non-toxic metabolite of disulfuram (i.e. ANTABUSE), has been shown to be an effective chelating agent against heavy metal poisoning (12). Borch and Pleasants showed in 1979 that DDTC could ameliorate the dose-limiting nephrotoxicity of Cisplatin in rats (13). These investigators subsequently showed that the cytotoxic effect of cisplatin was not compromised by the simultaneous use of DDTC (14). To test the hypothesis that DDTC could possibly ameliorate Cisplatin toxicity without altering its cytotoxic effect, a prospective randomized trial was conducted in patients with recurrent SCCHN that specifically evaluated the effects of DDTC on response, toxicity and pharmacology of Cisplatin.

MATERIALS AND METHODS

Between June 1985 and April 1987, 60 patients with recurrent SCCHN were randomized to receive either regimen A) Cisplatin (DDP) 100-120 mg/m^2 over 1 hour on day 1 plus 5-Fluorouracil (5-FU) 1000 mg/m^2 over 24 hours by continuous intravenous infusion on days 1-5, or regimen B) DDP/5-FU (as in A) plus DDTC 600 mg/m^2 over 30 minutes exactly 30 minutes after DDP infusion and DDTC 200 mg/m^2 intravenous push on days 8 and 15. Each cycle was repeated every 3 weeks. Response and toxicity were evaluated following each course of treatment. Standard criteria for major response were applied. The WHO toxicity scale was applied to grade toxicity. Pharmacology studies were performed on 11 patients, 5 Group A and 6 Group B; and the unbound platinum species was measured, with determination of mean residence time, area under the curve, total body clearance, and volume of distribution.

PATIENT CHARACTERISTICS

The following patient characteristics in both groups were equally balanced: Number of patients, sex, age, Zubrod performance status, primary site, previous treatments, and site of disease (Table 1).

RESULTS

The number of patients for Group A vs Group B were 31 vs 29; Inevaluable, 2 vs 2; Too early to evaluate, 1 vs 0; Evaluable, 28 vs 27. The overall response rate for Group A was 39% (11/28), with a complete response rate of 7% (2/28). Group B had an overall response rate of 29% (8/27), with a complete response rate of 7% (2/27). The difference between the groups was not statistically significant, P=0.37. The overall response rate for both groups combined was 34% (19/55). Median survival for Group A was 9 months, compared to 10 months for Group B, P=0.68 (Table 2).

TOXICITY

Renal toxicity for the two groups was based upon the cumulative Cisplatin dose (mg/m^2)(range) for Group A 360(100-720) vs Group B 300(100-820); number of patients was 30 vs 29; mean baseline serum creatinine (mg/dl), 0.90 vs 0.85; mean maximum serum creatinine (mg/dl), 1.50 vs 1.45; number of patients with >50% increase in baseline serum creatinine during any course, 19(63%) vs 17 (58%); number of patients with any increase in serum creatinine to 1.5- 2.0 mg/dl (%) or >2.0 mg/dl was 7(23%) vs 5(17%) and 6(20%) vs 3(10%), respectively (Table 2).

Other toxicities: nausea + vomiting was 27(90%) vs 26(89%); stomatitis, 25(83%) vs 24(82%); diarrhea, 20(66%) vs 13 (44%); leukopenia, 26(86%) vs 24(82%); thrombocytopenia, 14(46%) vs 11(37%); anemia, 23(76%) vs 27(93%); neurotoxicity, 11(36%) vs 10(34%); and clinical ototoxicity, 9(30%) vs 7(24%).

PHARMACOKINETICS OF CISPLATIN

Mean residence time (amount of time required to eliminate 63% dose)(min) was 33.4 $\pm$ 4.4 vs 45 $\pm$ 14.2; area under the curve ($\mu g.min.ml^{-1}$), 214.8 $\pm$ 62.6 vs 200.3 $\pm$ 59.5; total body clearance ($ml.min^{-1}/m^2$), 275.0 $\pm$ 73.8 vs 343.6 $\pm$ 117.5; volume of distribution, 9.1 $\pm$ 2.4 vs 16.5 $\pm$ 10 (Fig. 1).

TABLE 1. PATIENT CHARACTERISTICS

	Group A (CisDDP + 5-FU)	Group B (CisDDP + 5-FU + DDTC)
Patients entered	31	29
Sex: Male/Female	25/6	25/4
Age range (median)	27-75(57)	51-81(62)
Zubrod performance status 1	24	16
2	7	13
Primary site:		
Oral cavity	4	10
Oropharynx	7	10
Hypopharynx	3	2
Nasopharynx	4	2
Larynx	7	3
Paranasal sinus	2	0
Skin	3	1
Unknown	1	1
Previous treatments:		
Surgery alone	3	4
Radiation alone	11	8
Surgery + radiation	16	16
Previously untreated	1	1
Prior chemotherapy	5	5
Site of disease:		
Local + regional recurrence	20	16
Local + distant metastasis	3	8
Distant metastasis only	7	3
Local + regional + distant metastasis	1	2

TABLE 2.

	Group A (CisDDP + 5-FU)	Group B (CisDDP + 5-FU + DDTC)
Total no. of pts.	31	29
Inevaluable	2	2
Evaluable	28	27
CR	2(7%)	2(7%)
PR	9(32%)	6(22%)
CR + PR	11(39%)	8(29%)
Median survival	9 mos	10 mos
Toxicities:		
Renal		
Median cumulative dose CisDDP(mg/m^2)	360	300
Mean max serum creat (mg/dl)	1.5	1.45
No. of pts. with		
serum creat 1.5-2.0	7(23%)	5(17%)
or >2.0	6(20%)	3(10%)
Nausea + vomiting	27(90%)	26(89%)
Stomatitis	25(83%)	24(82%)
Diarrhea	20(66%)	13(44%)
Leukopenia	26(86%)	24(82%)
Thrombocytopenia	14(46%)	11(37%)
Anemia	23(76%)	27(93%)
Neurotoxicity	11(36%)	10(34%)
Ototoxicity	9(30%)	7(24%)

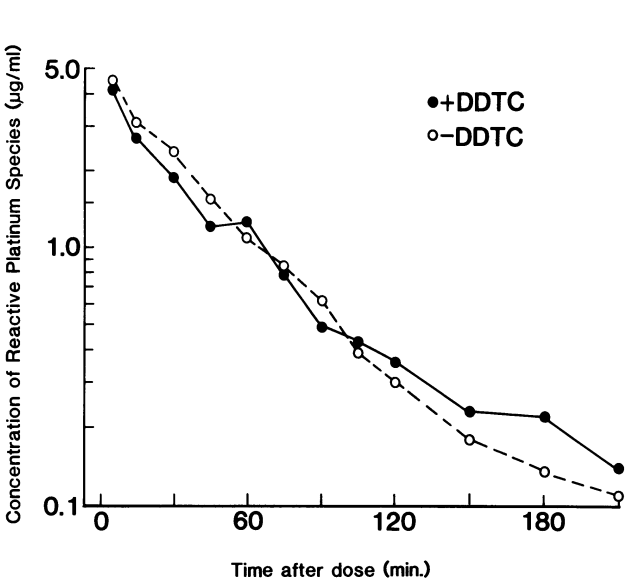

Fig. 1

CONCLUSIONS

1. The present dose and infusion schedule of DDTC did not reduce DDP-induced toxicities.
2. DDTC did not alter the pharmacokinetic parameters of ultrafilterable Cisplatin.
3. The difference in clinical response and median survival between the groups was not statistically significant.

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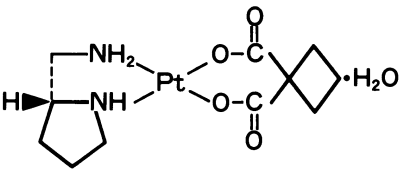
CLINICAL PHARMACOKINETICS OF (R)-(-)-1,1-CYCLOBUTANE-DICARBOXYLATO-(2-AMINOMETHYLPYRROLIDINE) PLATINUM(II) (DWA2114R)

H. Majima and H. Kinoshita

INTRODUCTION

(R)-(-)-1,1-Cyclobutanedicarboxylato(2-aminomethylpyrrolidine) platinum(II)(DWA2114R) (Fig. 1), second generation platnum (Pt) compound, has been advanced to clinical trial because of wide spectrum of activity, lack of renal toxicity and less myelotoxic than CBDCA in preclinical studies. In animals, the dose limiting toxicity was myelosuppression, especially thrombocytopenia (unpublished data). In a phase I trial of very widely spaced doses in which DWA2114R was administered as a 20 min i.v. infusion without pretreatment hydration, maximum tolerated dose was found to be 800 mg/m^2 with myelosuppression, particularly leukopenia, being the dose limiting toxicity. There are no renal- and neuro-toxicity except mild nausea and vomiting. The pharmacokinetic studies of DWA2114R during the phase I trial were studied in 6 patients who received from 40 to 800 mg/m^2. Total Pt and filterable Pt in plasma were measured. In this paper we shall briefly review our experience to date with the clinical pharmacokinetics of DWA2114R.



DWA2114R

$C_{11}H_{18}N_2O_4\ Pt \cdot H_2O$

455.37

Fig. 1. Structure of DWA2114R

MATERIALS AND METHODS

Sample Collection.

Blood (5 - 10 ml) was collected at pre-, mid-, end- infusion and at 2, 4, 6, 8, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 24 hr after the end of infusion. Blood was centrifuged immediately and plasma separated and stored at -70 °C until assay of Pt.

Pt Estimations.

Plasma sample (400 μl) were lyophilized, and digested with 5 ml of 6% HNO_3. The residue was dissolved in 3-4 ml of 2% HNO_3. Aliquot of this digest was used for total Pt estimations by flameless atomic absorption spectrophotometry (FAAS). The remaining plasma was ultrafiltered using Amicon MPS ultrafiltration membranes. A 300 μl aliquot was used for the measurement of filterable Pt species by FAAS. The remaining plasma ultrafiltrate was used for the measurement of unchanged DWA2114R. Urinary Pt was measured by FAAS as same as plasma.

Measurement of Unchanged DWA2114R.

Unchanged DWA2114R was measured by high pressure liquid chromatography (HPLC) with UV detection at 220 nm. Separation of DWA2114R in plasma filtrate was carried out on a Waters Associated μ Bondapak Phenyl Column or C_{18} Column with methanol/0.02M Na_2SO_4 (5:95) as the mobile phase. A chromatogram of the plasma of a patient before and after receiving DWA2114R, using this procedure is shown in Fig. 2. Separation of DWA2114R and other Pt species in urine was carried out by the HPLC as described above.

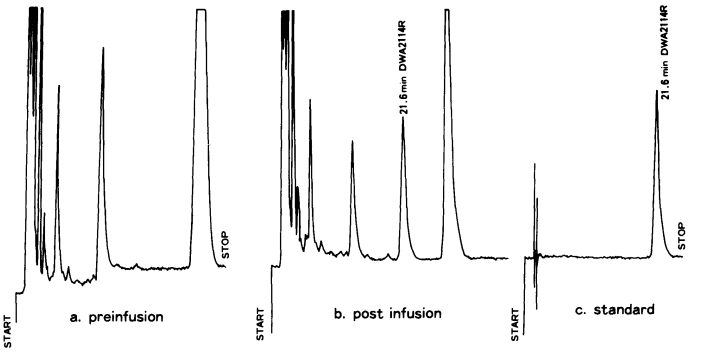


Fig.2. HPLC profiles of DW 2114 R.

Data Analysis.

Plasma levels of total Pt was fitted to a three compartment open model. The pharmacokinetic parameters were calculated by method of residuals.

RESULTS

Plasma decay profiles of the 2 Pt species namely, total Pt and filterable Pt in a patient receiving DWA2114R (500 mg/m^2) without pretreatment hydration is shown in Fig. 3. The pharmacokinetic parameters derived for each of these species is given in Table 1. The pharmacokinetic characteristics of each of the species is described below.

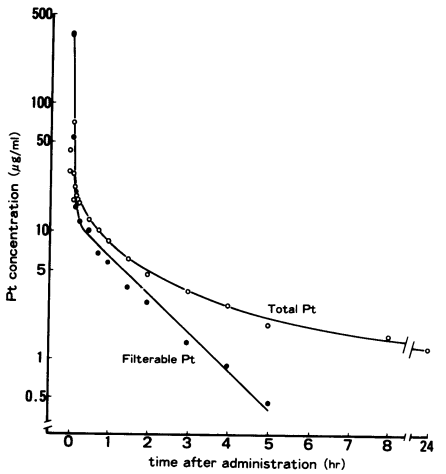


Fig. 3. Plasma concentration for total platinum and filterable platinum in one patient after administration of 500 mg/m^2 of DWA2114R by rapid infusion

Total Pt.

Plasma decay of the total Pt showed triphasic at all the doses studied (40 - 800 mg/m^2). It was a very short $t_{1/2}\alpha$(median 5.1 min), a short $t_{1/2}\beta$(median 1.33hr) and a long $t_{1/2}\gamma$(median 20.3 hr) which appears to be a result of the plasma protein binding of DWA2114R. These parameters did not change in the range of dose from 40 to 600 mg/m^2 and an AUC of the total Pt correlated with the dose. At a dose of 800 mg/m^2, however, the AUC increased markedly.

Table 1. Pharmacokinetic parameters of DWA2114R

Patients	Dose(mg/m²) (mg/body)	S(m²)	C_0(pt·μg/ml)		$t_{1/2}$			AUC (μg/ml·hr)	Free Pt (%)	Clrenal (ml/min)	Clcreat (ml/min)	Urinary Excretion 0~24hr(%)
			Calculated	Observed	α(min)	β(hr)	γ(hr)					
1 R.A.♀	40 (59)	1.46	3.54 2.89	3.89 3.20	4.1 4.7	1.02 1.53	12.4	13.35 3.88	29.1	40.6	26.4	37.4
1' R.A.♀	160 (240)	1.46	9.92 6.82	31.95 23.50	5.0 17.0	0.65 1.58	13.9	60.23 18.17	30.2	30.8	29.4	32.7
2 J.K.♂	240 (310)	1.29	32.07 15.97	36.85 25.56	8.4 7.2	3.39 1.68	—	44.40 17.13	38.6	69.6	34.7	53.9
3 U.Y.♀	400 (480)	1.20	19.75 4.81	87.79 25.11	6.0 10.1	1.13 1.76	25.6	110.41 7.80	7.1	221.6 (0~8hr)	57.9	48.8 (0~8hr)
4 S.S.♀	500 (665)	1.33	248.87 114.73	42.80* 29.14**	1.6 2.0	0.94 1.01	35.0	125.27 22.57	18.0	127.1	70.3	60.5
5 T.K.♀	600 (762)	1.27	— —	— —	— —	— —	—	— —	—	—	34.9	50.6
6 M.S.♀	800 (1100)	1.38	55.19 49.28	54.33 51.96	5.7 8.7	0.86 1.39	14.5 7.67	295.87 97.77	32.7	83.0	57.8	70.7

Filterable Pt.

The pharmacokinetics of filterable Pt showed a biphasic decay with $t_{1/2}\alpha$ (median 8.3 min) and the $t_{1/2}\beta$ (median 1.49 hr) the latter being approximately equal to $t_{1/2}\beta$ of total Pt. These $t_{1/2}$ did not change in the range of dose from 40 to 600 mg/m^2. The filterable Pt AUC following DWA2114R administration represents about 26% of the total Pt AUC. At a dose of 800 mg/m^2, the AUC increased as same as total Pt.

Urinary excretion of Pt.

The urinary excretion of Pt was rapid initially, markedly by 24 hr, shown in Fig. 4. An overall excretion of 32.7-70.7% in the first 24 hr was found in a total of 5 patients studied. One patient with the urinary excretion of 32.7% had reduced creatinine clearance (Ccr), but another patient with urinary excretion of 60.5% had normal Ccr. The relationship between Ccr and urinary excretion of Pt administered as DWA2114R is shown in Fig. 5. Virtually Ccr correlated with urinary excretion of DWA2114R Pt.

The clearance of Pt, however, exceeds Ccr suggesting that DWA2114R involves other compensatory renal mechanism.

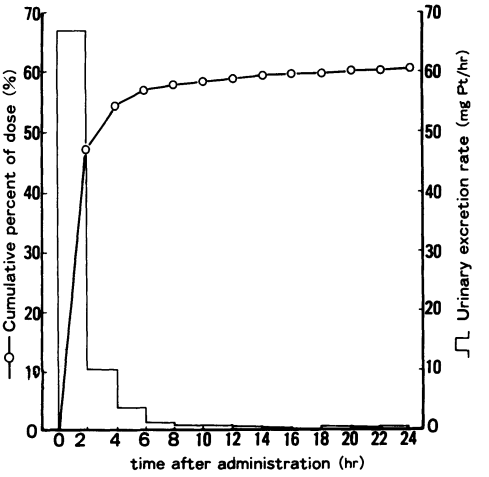


Fig. 4. Urinary excretion of platinum in one patient after administration of 500 mg/m^2 of DWA2114R by rapid infusion
0-4 hr: 54.2%
4-24 hr: 6.3%

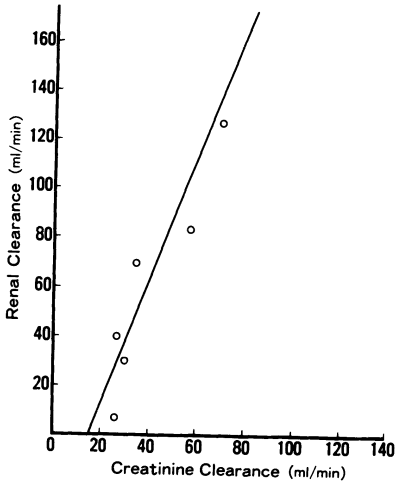


Fig. 5. Correlation between creatinine clearance and filterable platinum clearance

Toxicity.

The relationship between AUC of plasma filterable Pt and the percentage of reduction in leukocyte/platelet count is shown in Fig. 6. Although AUC of plasma filterable Pt correlated with the percentage of reduction in leukocyte count, that of plasma filterable Pt did not correlate with platelet count. These findings are different from those of CBDCA, shown by Egorin et al.(1).

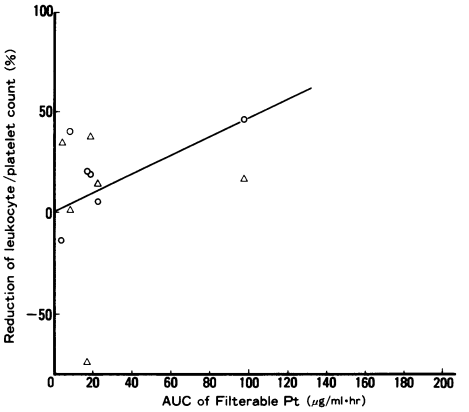


Fig. 6. Correlation between the reduction of leukocyte (o) and platelet (Δ) count and AUC of filterable Pt

DISCUSSION

DWA2114R has been introduced into clinical trial as a less toxic alternative to cisplatin. The pharmacokinetic properties of DWA2114R are clearly different from those of cisplatin(2) but similar to CBDCA(3,4) and are summarized in Table 2.

DWA2114R in plasma after 20-min infusion showed a triphasic decay in total Pt, but a biphasic one in filterable Pt by these analyses. A short distribution phase was seen in all patients studied.

An AUC of plasma total and filterable Pt correlated linearly with doses ranged from 40 to 600 mg/m^2, at a dose of 800 mg/m^2 AUC increased markedly.

The data presented here demonstrate that renal function impairment (as indicated by Ccr) was associated with a decrease in total body clearance of filterable Pt and the reduction in leukocyte count seen after administration of DWA2114R. It is noteworthy that an AUC of plasma total and filterable Pt correlated with the reduction in leukocyte count. These findings has not been yet observed in second generation Pt compounds, particularly CBDCA and CHIP.

Table 2. Comparative pharmacokinetics of DWA2114R, CBDCA, and CDDP

	Total platinum $t_{1/2}\alpha$(min)	Total platinum $t_{1/2}\beta$(hr)	Total platinum $t_{1/2}\gamma$(hr)	Free platinum $t_{1/2}\alpha$(min)	Free platinum $t_{1/2}\beta$(hr)	% of protein bound(~4hr) postinfusion	24-hr urinary platinum excretion (% of dose)	Free platinum renal CLR/GFR	Free platinum plasma CLR(ml/min)	In vitro free platinum $t_{1/2}$ (hr)
DWA2114R	1.6–8.4	0.65–3.39	12.4–35.0	2.0–17.0	1.01–1.76	39.4–86.2	32.7–60.5	–	30.8–127.1	14.5**
CBDCA*	98	6.7–>24	–	87	5.9	24	65	0.7	123	30
CDDP*	18–37	44–190	–	22–78	Not seen	>90	16–35	0.9–1.9	400–600	1.5–3.7

*Harland,S.J.,Cancer Res. 44 : 1693–1697, 1984 **healthy volunteer

There were less renal toxicity in 6 patients including 3 ones who were less than 50 ml/min in Ccr. Therefore, DWA2114R seems to be less nephrotoxic agent, as compared with CDDP and CBDCA.

From the present study it might be possible to adjust the dose according to the Ccr of each patient to give a specified reduction in leukocyte count.

It is notable that a similar relationship of renal function, drug clearance, and toxicity had been observed with CBDCA(4). These findings emphasize the value of making such clinical pharmacological measurements early in the trial of new antineoplastic agents.

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COMBINATION OF CISPLATIN AND 5-FLUOROURACIL IN THE TREATMENT OF ADVANCED BREAST CANCER

P. Pronzato, D. Amoroso, G. Bertelli, P. Gallotti and R. Rosso

INTRODUCTION

Combination of Cisplatin (CDDP) and 5-Fluorouracil (5-FU) has been shown to have an encouraging activity both in rodent tumor systems (1), and in the treatment of various solid tumors (2), particularly in head and neck carcinoma, testicular and ovarian cancer, and more recently, in bladder carcinoma.

Literature's review on use of CDDP in the treatment of metastatic breast carcinoma has shown its efficacy particularly in untreated patients (pts) either as single agent or in combination with other drugs (Cyclophosphamide, Doxorubicin) (3,4).

Few data are presently available relating to the effectiveness of CDDP and 5-FU combination in the treatment of pts with advanced breast cancer refractory to conventional agent (5). Consequently, our Division carried out a study to evaluate the activity of this combination in pretreated metastatic breast cancer pts.

MATERIALS AND METHODS

Fifteen pts with histologic diagnosis of breast cancer and evaluable progressive disease on more conventional therapy, entered the study after informed consent. Patients were required to have normal renal, cardiac and hepatic function and Performance Status <3 (ECOG scale). Dose schedule used was the following: CDDP (20 mg/sm for 5 consecutive days) was administered after prehydration (40 mg of furosemide + 500 ml of saline infusion + 5 mEq/L of KCl over a 30 minute infusion). CDDP was given if diuresis was >200 ml/30 minutes. Posthydration consisted of 1.5 L of NaCL solution + 5 mEq/L of KCl. 5-FU (200 mg/sm) was administered by i.v. bolus after the end of posthydration. Methylprednisolone was given at the time of CDDP infusion for nausea and vomiting control. This schedule was performed every 3 weeks. Response and toxicity were assessed according to WHO criteria.

Characteristics of pts were: all postmenopausal, median age 55 years (range 33-72), dominant site of metastases: soft tissue 3 pts, bone 2, viscera 10 (liver 3 and lung 7); all pts had large metastatic involvement and had previously failed to a median of 3 regimens of chemotherapy (range 1-4). The median of courses carried out was 2 (range 1-6).

RESULTS

Out of 15 entered pts, 14 are evaluable for response and toxicity: one pt had early death. Two pt achieved a Partial Response (PR): one of lung metastases, lasting 7 months, and the other of liver lesions (with a Complete Response of soft tissues lesions), of 7 month-duration. 5 pts had a Stable Disease (SD) and 7 progressed. One other pt, whose response was classified as SD, obtained a noteworthy reduction of CEA levels after 4 courses of therapy (from 2400 mg/ml to 470 mg/ml). Median progression free survival for PR and SD pts was 6 months, with a median survival from beginning of therapy of 8 months.

Major toxicities observed were: nausea and vomiting, grade II-III, in 80% of pts; leukopenia, grade II-III, in 70% of pts; 1 pt experienced hair loss (grade II), 1 pt developed mucositis (grade II). No renal toxicity, as increase of BUN or creatinine, was observed.

DISCUSSION

Little is known regarding the activity of CDDP in previously untreated pts with metastatic breast cancer: Kolaric et al. (4) in a Phase II study comparing CAP (Cyclophosphamide, Adriamicin, Cisplatinum) versus CMFVP (Cyclophosphamide, Methotrexate, 5-Fluorouracil, Vincristin, Prednisone) combination obtained an

interesting 75% of objective response (OR) in CAP regimen versus 44% in CMFVP ($p<0.001$). Previously, Kolaric himself achieved a 54% of OR in 35 pts with CDDP at the dose of 30 mg/sm i.v. by a 4-hours infusion for 4 days (3). These data demonstrate that CDDP alone or in combination is active in management of untreated metastatic breast cancer pts: unfortunately the same does not occur in pretreated pts. In fact, a revision of available data showed a failure of CDDP alone or with the same drugs used in combination regimens for untreated pts (3,4). The association of CDDP and 5-FU appeared to increase the usual clinical response achieved with single agents in esophageal, head and neck cancer, non small cell lung cancer, ovarian and testicular cancer and bladder carcinoma. This effectiveness in solid tumors encouraged us to test efficacy of CDDP and 5-FU combination in metastatic breast cancer also.

Even if response rate observed with this combination was low, a modest activity is demonstrated in far advanced pts with acceptable toxicity: in particular, the use of pre- and posthydration avoided CDDP-related nephrotoxicity.

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Istituto Nazionale per la Ricerca sul Cancro

V.Le Benedetto XV, 10

16132 Genova - Italy

ANTITUMOR EFFECTS OF HIGH-DOSE CISPLATIN IN HYPERTONIC SALINE AGAINST HUMAN OVARIAN TUMORS HETEROTRANSPLANTED IN NUDE MICE

M. Sawada

INTRODUCTION

Cisplatin (CDDP) is among the most active chemotherapeutic agents available for the treatment of ovarian cancer (1). However, CDDP is a toxic drug causing emesis, renal failure, hearing loss, neuropathy and anemia. Efforts to explore methods of reducing its nephrotoxicity and increasing its therapeutic index have continued. Recently, Litterst showed that preparation of CDDP in vehicles containing hypertonic saline significantly protected animals against the toxicity without altering the antitumor effect of the drug (2). Consequently, this approach has been introduced into the clinic where CDDP has been administered in a 3% NaCl-containing solution (3). In this paper, the antitumor effects of high-dose cisplatin in hypertonic saline against human ovarian tumors heterotransplanted in nude mice are examined.

MATERIALS AND METHODS

The human ovarian tumors (OVA-1,-2,-3,YST-1,-2,-3) used in this study were established by inoculation of fresh tumor tissues into BALB/C female nude mice in our laboratory, as previously described (4-7). The tumors were cut into small pieces (2-4 mm^3) in ice-cold Eagle's minimum essential medium and transplanted s.c. into nude mice by trocar.

CDDP was supplied by NIPPON KAYAKU Company. For standard dose administration, 2 mg/kg & 6 mg/kg of CDDP was dissolved in

0.2 ml 0.9% NaCl and for high dose administration 12 mg/kg of CDDP was dissolved in 0.2 ml 3% NaCl. Two mg/kg, 6 mg/kg and 12 mg/kg of CDDP were administered i.p. into tumor-bearing nude mice three times with intervals of 4 days. Control mice were injected i.p. with 0.2 ml 0.9% NaCl.

When the tumors became palpable and were growing progressively, experimental mice were randomized into test groups of 5-10 mice (one tumor each). The size of the implant was measured with slide calipers twice a week, and the volume(V), in mm^3, was calculated by the formula described by Houchens et al. (8) : $V=W^2xLx1/2$, where W and L are the width and length in mm. For comparison with different groups, the relative tumor volume (RV) for each group was calculated from the formula RV=Vi/Vo, where Vi=the mean tumor volume at any given time and Vo=the mean initial tumor volume when treatment was begun. T/C (ratio of RV for treated mice to RV for control mice, multiplied by 100) was calculated at each measurement.

RESULTS AND DISCUSSION

Response to chemotherapy is shown as relative tumor volume in Fig. 1. Summary of the antitumor effect of CDDP on human ovarian tumors heterotransplanted in nude mice is shown in Table 1.

Table 1. Summary of the antitumor effects of CDDP

Tumor line	Dose (mg/kg)	T/C	(day)
OVA-1	2	62	(19)
	6	51	(16)
	12	26	(19)
OVA-2	2	35	(17)
	6	28	(24)
	12	15	(24)
OVA-3	2	24	(26)
	6	11	(28)
	12	7	(21)
YST-1	2	24	(29)
	6	7	(25)
	12	14	(28)

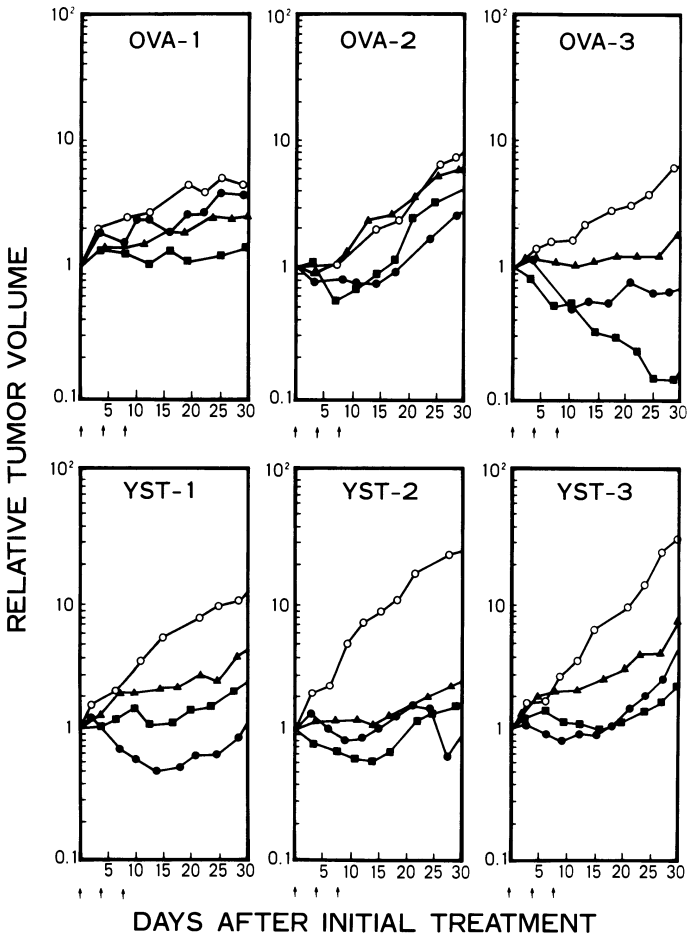

Fig. 1. Response to chemotherapy (shown as RV) of human ovarian tumors in nude mice.

O = controls, ▲ = CDDP 2 mg/kg, ● = CDDP 6 mg/kg, and ■ = CDDP 12 mg/kg.
Arrows indicate injection of CDDP.

YST-2	2	23	(29)
	6	3	(27)
	12	12	(28)
YST-3	2	37	(26)
	6	11	(27)
	12	10	(23)

The human tumors except for OVA-1 in nude mice responded well to CDDP at various doses. The antitumor activity depended on the dosage of the drug. The most interesting result was that high-dose CDDP succeeded to show antitumor effect against mucinous cyst-adenocarcinoma (OVA-1), which was refractory to standard dose CDDP. A comparison of body weights of tumor-bearing nude mice before and on Day 30 of treatment did not show any appreciable treatment-related changes. Our results suggest that if high-dose CDDP can be successfully administered, it may lead to increased response rates compared with standard-dose CDDP.

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IP VERSUS *IV* PHARMACOKINETICS IN PATIENTS USING RADIOLABELLED PARAPLATIN

H.L. Sharma, N.D. Tinker, C.A. McAuliffe, M.J. Lind, D.B. Smith and D. Crowther

Introduction

Effectiveness of an anticancer drug lies in its ability to reach the site of action and its prolonged presence there in concentration sufficient to act against the tumor cells. Usually the toxicity of such compounds against normal cell lines, whole organs or other biochemical processes is the limiting factor and can dictate the maximum dose that can be administered to a patient. Simple physical considerations such as local application of the drug can enhance the tumor exposure, and therefore may increase its therapeutic effectiveness.

The majority of epithelial ovarian tumors are confined to the abdominal cavity and have responded to therapy by Cisplatin as well as Paraplatin. The intraperitoneal administration of these agents may well be more effective in such cases from physical considerations alone. For, in addition to the therapeutic advantage, there may be a reduction in the systemic toxicity since, a slow absorption of these drugs from the pertitoneal cavity may keep the plasma concentration of the compounds low.

At present in the department of Medical Oncology a study is in progress into the use of intraperitoneal Iproplatin and Paraplatin in patients with minimal residual disease following systemic chemotherapy. A pharmacokinetic investigation has been undertaken in parallel to this clinical study in order to measure the clearance rates of Cisplatin Iproplatin and Paraplatin from plasma, red blood cells and excretion in urine fractions upto 72 hours post administration of the drugs given intravenously or intraperitoneally.

Materials and methods

For these measurements, tracer quantities of radioactively labelled compounds were mixed with the therapeutic doses prior to administration. A gamma-emitting radionuclide can be measured quantitatively in body fluids such as blood, plasma and urine without the necessity of any further processing. The radionuclide of Pt used in these investigations is Pt-195m; it has a half-life of 4.1 days and decays predominantly by internal transition to stable Pt-195. The main gamma-rays of the Pt-195m have 99 keV energy and are emitted in 11% of its decays in addition to the Pt X-rays of around 66 keV and 76 keV.

Platinum-195m is produced by neutron irradiation of enriched Pt-194 (95.06% purchased from Oak Ridge National Laboratory, Oak Ridge, Tn, USA) at a neutron flux of 2×10^{14} n sec^{-1} cm^{-2} for upto 4 days. Typically a 50 mg sample of Pt-194 is irradiated and used for the synthesis of the compounds. Sometimes, the irradiated material is mixed with natural Pt to synthesise low specific activity compounds. A detailed procedure for the microscale synthesis of these drugs has been published (Ref.1). However a few modifications have been made to a few steps of the synthesis procedure either to reduce the time or increase the efficiency of these stages. A quality control of the final products is carried out by thin layer chromatography (TLC) prior to their use in human subjects.

Experimental protocol

The compounds (10-20 mg) are dissolved in sterile saline or water for injection (B.P.) and millipore filtered (0.22 μ) in to 10 ml sterile injection vials. The radioactivity content of these vials is accurately measured. These doses are then mixed with the therapeutic doses and are infused intravenously or into the intraperitoneal cavity over a period of 60 minutes.

Blood samples, each about 5 ml, are taken at 5 minutes, 15 minutes, 30 minutes, 60 minutes, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 48 hours and 72 hours post administration. Urine fractions are collected at 1 hour, 6 hours, 12 hours, 24 hours, 48 hours and 72 hours following the drug infusion.

In addition to these, for the patients with intraperitoneal infusion, the ascite fluid samples are withdrawn from the intraperitoneal cavity at various times upto 4 hours, at which point the contents of the adbominal cavity are removed altogether.

Concurrent with these measurements, a complete biochemical profile of the patient is carried out prior to and several days after the treatment. Parameters such as creatinine levels, creatinine cleavance rates, lymphocyte counts etc are recorded.

Measurements

Concentrations of the radioactive label are measured by gamma counting. In order to minimise errors in these measurements, a method of parallel standards is employed, i.e. in parallel to the dose, a standard is made and activity in the two measured in an identical manner. While the dose is given to the patients, the standard is diluted by a known factor to be counted in parallel to the blood, plasma and other fluids. Concentration measurements are carried out in whole blood, plasma, Red blood cells, urine and intraperitoneal fluids.

Patient Details

IV

4 patients were treated with intravenous paraplatin. All patients had a histological diagnosis of epithelial ovarian cancer. 3 were receiving paraplatin together with cyclophosphamide as initial therapy following debulking surgery. Each of these patients received a maximum of 6 cycles of chemotherapy after which they were restaged. The 4th patient received single agent paraplatin having relapsed epithelial ovarian cancer following treatment with surgery and cyclophosphamide. The patients creatinine clearances and nadir white blood cell counts are recorded in Table 1.

TABLE 1

IV Patient Details

PATIENT	CREATINE CLEARANCE (MLS/MIN)	NADIR WBC (X 10^9/L)
EK	54	0.6
S.O.	106	7.3
C.M.	218	2.7
M.A.*	32	7.4

* 50% REDUCATION IN DOSE

IP

3 patients were treated with intraperitoneal paraplatin at a dose of 400 mg/m^2, two of these patients had evidence of minimal residual disease following treatment with initial debulking surgery and combination chemotherapy consisting of 6 cycles of cyclophosphamide and a platinum analog. The 3rd patient had relapsed following surgery, first and second line chemotherapy but the major component of her disease was ascites and she was therefore considered suitable for treatment by this route. The procedure for intraperitoneal administration of the drug was as follows. A temporary peritoneal dialysis catheter was inserted midway between the symphisis pubis and the umbilicus. Radiopaque dye was infused and a CAT scan performed to assess distribution of fluid. In all 3 patients distribution was satisfactory. The paraplatin was infused in 2L of 5% Dextrose and left in the abdominal cavity before being drained out. A maximum of three cycles of IP treatment were given to each patient.

RESULT

i) Blood clearance

Blood clearance of the tracer followed a biexponential pattern in the case of intravenous infusion as well as intropertioneal infusion. Since both the infusions were carried out over a period of 60 minutes, the blood concentration is expected to be at its highest at 60 minutes in IV cases whereas for the IP administered patients, the concentration in the blood will keep on rising as the compound is progressively absorbed from the abdominal cavity into the blood. Table 2 shows the half-times of the clearance phases as well as the fractions of the tracer cleared by each phase.

TABLE 2

Blood Clearance of Pt-195m-paraplatin

	IV (4 Patients)	IP (3 Patients)
$t_{\frac{1}{2}}$	3.34 ± 0.34 Hrs	2.46 ± 0.52 Hrs
A%	80.9 ± 12.2%	84.9 ± 4.2%
$t_{\frac{1}{2}}$	116.8 ± 14.4 Hrs	114.5 ± 31.4 Hrs
B%	19.1 ± 12.2%	15.1 ± 4.2%

ii) Urinary Clearance

The total urinary clearance up to 72 hrs in IP patients was 46.9 ±14.0% of the administered dose. This figure should be seen in the context that after four hours following IP infusion, the residual activity from the abdominal cavity is emptied. A second interesting feature of the urinary clearnace in IP patients is that a large fraction of the excreted drug came out not in the fractions accumulated up to 6 hours but over the first 12 hours. This is of course expected since a slowing down effect of absorption from the abdominal cavity should manifest in a delayed urinary clearance.

In IV infused patients urinary clearance showed a normal pattern of elemination in two patients, i.e. majority of the label being excreted during the first 6 hours followed by a slow clearance. However two patients who had reduced renal function exhibited a completely unexpected pattern of urinary clearance as shown in Figure I.

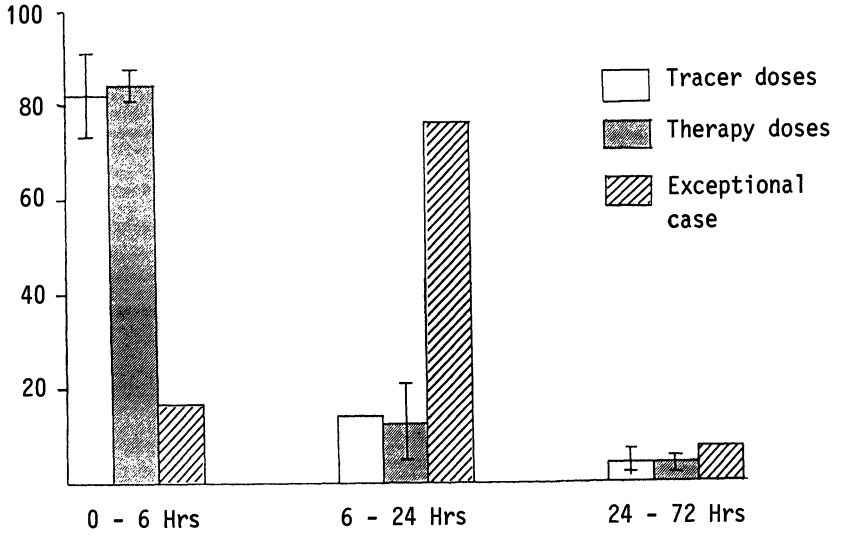

Fig.1: Urinary excretion of platinum following IV administration of Paraplatin expressed as percentage of the total excreted over 72 hour period.

iii) Concentration in Ascite Fluid

The fluid samples from the IP cavity were taken from the patients infused introperitioneally with the drug. The tracer concentration was measured in these samples taken up to 4 hours, after which the contents of the adbominal cavity were removed and therefore no further measurement was deemed necessary. From these measurements the area under the curve (concentration in ascite fluid Vs time) was calculated and compared with the similar area under the blood concentration curve. Two ratios have been computed from these measurements (1) the ratio of AUC up to 4 hours on ascite fluids and AUC upto the same time in blood and (2) the ratio of AUC upto 4 hours in ascite fluid and AUC upto 72 hours in blood.

These are presented in Table 3.

TABLE 3

Area Ratios (Ascite Fluid Vs Blood) in IP patients

Patient	Ratio 1	Ratio 2
E.C.	45.6	3.56
F.C.	29.6	3.23
S.C.	61.8	8.0
Average	45.6 ± 16.1	6.0 ± 2.7

Ratio 1 = $\frac{\text{AUC (Ascite fluid 0-4 Hr)}}{\text{AUC (Blood 0-4 Hr)}}$; Ratio 2 = $\frac{\text{AUC (Ascite 0-4 Hr)}}{\text{AUC (Blood 0-72 Hr)}}$

DISCUSSION

Blood clearance data obtained in this pharmacokinetic study is consistant with our previous measurements (Ref 2) carried out with tracer quantities of the radiolabelled paraplatin and injected intravenously as a bolus. Bolus injection produced clearance curves with three phases, a very fast phase with a $t_{\frac{1}{2}}$ = 10.8 minutes followed by an intermediate phase of $t_{\frac{1}{2}}$ = 2.5 hours and finally a slow phase with half-time of 125 hours. In this present study, the labelled tracer was administered mixed with the therapeutic dose and secondly was infused slowly in 60 minutes. Of course the slow infusion process prevents the observation of the very fast phase of blood clearance. The remaining two phases have half-clearance times which are consistent with the bolus administration. The ratio of activities excreted by these two phases is also consistent. These observations coupled with the similarity of results in urinary clearance point towards an important conclusion that the tracer quantities and the therapeutic doses of paraplatin show a similar pattern of elemination from blood and body.

The total amount of the tracer excreted in IP patients was about half that of IV patients. This is an entirely expected result since at 4 hours the drug that has not been absorbed from the abdominal cavity is removed.

The interpretation of area ratios is controversial. The area under the ascite fluid is calculated only up to 4 hours whereas the area under the blood concentration curve can be taken up to 4 hours, 72 hours (the maximum observation time in our 'investigation') or up to infinity by mathematical extrapolation. Which ratio represents meaningful numbers and therefore should be used when clinical significance is being derived? The traditional method, used by many authors, is to extrapolate the blood clearance curve to infinity. This method of computation assumes that the platinum compounds present in blood are therapeutically active with the same therapeurtic index at all times. This is an entirely unrealistic assumption particularly in view of the fact that the blood clearance has three phases and moreover, the slow clearance phase matches well with the slow urinary excretion, indicating the clearance of a metabolite. Thus in our view not all the compound present in the blood is equally active at all the times. Therefore the therapeutic advantage of the IP administered drug in the case of ovarian tumours should be represented by Ratio 1,which is 45.6 ±16.1 in our study. If, however, one assumes that at least a part of the drug present in the blood remains active against tumour cells then the number representing the therapeutic advantaged should lie between the two ratios i.e. 45.6 ±16.1 and 6.2 ±2.7.

Among the group of patients given the drug intravenously, two patients had an impaired renal function indicated by creatinine clearance of 54ml/min and 32ml/min. Both of these patients showed a delayed urinary clearance. The first patient developed severe neutropenia and thrombocylopenia resulting in septicemia requiring hospitalization and intensive supportive care. In view of these results, the dose administered to the second patient was reduced to 50%, and the blood profile was carefully watched during the post therapy period. Although several mathematical formulae have been proposed to calculate the drug doses from the creatinine clearance data, there is no final agreement among the clinicians. However, our observation points towards a relationship between delayed urinary clearance and myelotoxicity. Much more data is needed before a definite conclusion can be drawn.

The studies are continuing.

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D: Advances in Antitumor Research with Platinum and Non-Platinum Complexes; Development of New Metal Coordination Compounds: Synthesis and Characterization.

THE SEARCH FOR NEW PLATINUM ANTITUMOR AGENTS: PROGRESS, PROBLEMS AND PROSPECTS

E.W. Stern

It has now been some eighteen years since Rosenberg's momentous discovery of the antitumor activity of cisplatin (1). In the intervening period, cisplatin has become established as a leading anticancer agent and a great deal has been learned concerning its activity and toxicity as well as the nature of its interaction with its presumed biological target, DNA (2,3). During the same interval, several thousand platinum and, perhaps, a hundred or so other metal complexes have been synthesized and evaluated in various model tumor systems in hopes of discovering new drugs with improved properties. Of these, only a relatively small number have entered clinical trials and only one, carboplatin, has, to date, received regulatory approval and been commercialized. At this time it would be useful to review the requirements for an improved platinum drug, along with what has been acomplished so far in meeting these, what problems have been encountered, and what future progress is likely.

With respect to requirements, it is interesting that the clinically oriented goals of new compound work have remained relatively constant. They are:

1. Improved activity vs. cancers responding to cisplatin or, preferably activity vs. presently unresponsive cancers.
2. Lower acute and chronic toxicities, toxic side effects which are reversible and/or treatable, and if possible, dose limiting toxicities different from those of other drugs used in combination.

3. Lack of cross-resistance with cisplatin, i.e. activity in non-responding patients or in those who develop resistance.
4. Improved physical and pharmacological properties such as greater aqueous solubility, solution stability, and longer lasting action.

While cisplatin, alone or in combination with other agents, has had a revolutionary impact on the chemotherapeutic treatment of some cancers and its major toxicities can be ameliorated to some extent via various clinical strategies, there remains considerable incentive to develop new compounds capable of meeting one or more of these goals.

Given the number of compounds synthesized and evaluated, it would be surprising if some progress toward this end had not been made. Indeed, a number of second generation compounds are more soluble and stable than cisplatin and some have been found to be less toxic (at least with respect to nephrotoxicity) both in pre-clinical and clinical evaluations (3-5). Carboplatin is reported to be effective in some patients who do not respond to cisplatin (6) and a number of compounds (most notably those containing a 1,2-diaminocyclohexane ligand) are active both in vitro and in vivo against tumor cell lines which have been developed to resist cisplatin (7-11).

Progress toward compounds with improved activity or with activity against unresponsive cancers has been more elusive. Generally, compounds which are more stable and less toxic than cisplatin are also less potent, i.e., a greater amount of compound is required to achieve a comparable therapeutic effect. In many instances, this results in little or no net gain in therapeutic index, the ratio of toxic to active dose. A major problem in identifying compounds effective against a particular tumor type is the current lack of truly predictive models in which compounds can be evaluated.

In surveying progress or lack thereof in development of new, improved platinum species, one might well ask what progress has been made toward rationalizing this search? It is with respect to such

rational development that problems have arisen. Some of these difficulties, such as the lack of demonstrably predictive models, are not unique to platinum drugs.

Early work in Rosenberg's and other laboratories sought to establish structure-activity relationships (SAR's) by systematic replacement of the ammonia ligands in cisplatin with various neutral ligands and of the chlorides with other anionic groups (12). Problems with this approach which were immediately noted and which have not been satisfactorily resolved to this day are that SAR's depend on the screen, methodology (sites of administration of tumor and compound, dose schedule, number of tumor cells, etc.) and activity criteria employed; i.e., different screens and/or screeners can rank the same group of compounds differently.

Nevertheless, this early work established a set of guidelines or qualitative SAR's which established much of the tone of later synthetic efforts.

1. Compounds should have a cis configuration (two cis leaving ligands)
2. Compounds should be neutral
3. Non-leaving ligands should have a low trans effect generally similar to that of amines or ammonia, preferably with one hydrogen on the group directly bound to platinum.

In general, it was concluded that the variation of amines within complexes with the same leaving groups had some modifying effect on activity. The magnitude of the effect, however, was highly dependent on the particular screen employed. An effect on toxicity was also noted.

4. Leaving ligands should have moderate leaving ability, i.e., a "window of lability" centered on chloride. However, relatively stable bidentate ligands (e.g. dicarboxylates) also give rise to active compounds.

Since activity has been found among both kinetically labile and stable platinum complexes, this final point would appear to provide relatively little guidance. However, leaving group lability in aqueous solution does seem to correlate reasonably well with

toxicity, with compounds least stable to hydrolysis (e.g. nitrates, sulfates) being generally more toxic than those which hydrolyze very slowly (e.g. malonates) (13).

Attempts to quantitatively correlate biological activity with kinetic or thermodynamic parameters of various complexes have not succeeded (14).

Quite possibly, the above rules have the qualities of a self-fulfilling prophecy; i.e., close analogs of active compounds tend to be active. Indeed, the majority of platinum complexes made and tested are of the cis-PtA_2X_2 variety or are Pt(IV) analogs of these: cis-$PtA_2X_2Y_2$. All of the compounds which have entered clinical trials meet the criteria of the qualitative SAR's, with one possible exception, 4-carboxyphthalato-1,2-diaminocyclohexane-platinum(II) (15) which is solubilized in $NaHCO_3$ and, hence, could be considered charged.

Some exceptions to the classical qualitative SAR's have been reported. These include the anionic complexes, $K[Pt(NH_3)Cl_3]$ (13) and $K[Pt(t\text{-}BuNH_2)Cl_3]$ (16) as well as the novel neutral species, 3-aminoquinuclidiniumtrichloroplatinum(II) (17) which, if formulated correctly, contains a non-hydrogen bearing ring nitrogen bound to platinum. These materials display at least some in vivo activity against the L1210 leukemia. Other complexes lacking hydrogen on the binding nitrogen are cis-$[Pt(DMF)(2,4\text{-lutidine})]Cl_2$ (18) which is active vs the P-388 leukemia in vivo and some bipyridine complexes such as $[Pt(bipy)Cl_2]$ (19) and [Pt(bipy)(amino acid)]Cl (20), which have shown in vitro activity vs. the L1210 and P388, respectively. The bipy amino acid complex is formulated as a N-O chelate and therefore also represents a cationic Pt complex.

Less equivocal examples of active complexes which violate the classical qualitative SAR's are a series of cationic triamine Pt complexes, cis$[Pt(RNH_2)_2(L)Cl]Cl$, where (L) is pyridine, a substituted pyridine or a pyrimidine or purine, being developed in our laboratories. The majority of these stable, water soluble complexes show good activity in vivo against the S180 ascites and a few against the L1210. These results contrast with the previously reported inactivity of cationic triamines such as $[Pt(NH_3(3Cl]Cl$ (13) and [Pt(dien)Cl]Cl (12). There appears to be a requirement for

a cis disposition of the amine ligands and compounds containing chelating diamines examined so far are inactive. However, at this time, data are not sufficiently complete to support an explanation for the activity of this class of materials.

If empirically derived structure-activity relationships have, so far, not been successful in predicting which Pt species will be more active or have a different spectrum of activity than cisplatin, what of attempts to devise such structures based on a mechanism of action? This is an area that has received a great deal of attention. Much of the effort has been devoted to attempts to establish differences between the effects of active cis and inactive trans analogs on various biological processes at the cellular level. Most studies have concentrated on the binding of cis and trans-$Pt(NH_3)_2Cl_2$ to what is considered by the majority of workers to be the likely important cellular target, DNA (21).

Unfortunately, the differences in binding by DNA of cis and trans-$Pt(NH_3)_2Cl_2$ have been found to be more subtle than originally supposed. Both have been shown capable of forming intra as well as interstrand DNA and DNA-protein cross-links (22). While considerable progress has been made in determining which binding sites are involved and it has been shown that cis but not trans-$Pt(NH_3)_2Cl_2$ can bind to adjacent guanine bases on duplex DNA (22), the ultimate consequences of such binding to antitumor activity remain unclear. Thus the details of the mechanism of action of cisplatin are, as yet, not sufficiently well understood to provide guidance for the design of improved compounds. The assumption that DNA is the critical target for platinum compounds has given rise to attempts to make DNA more susceptible to attack by cis Pt species. Complexes have been prepared with ligands which are linked to known intercalators (23). While this novel approach is interesting, compounds prepared so far lack sufficient activity in initial screens to warrant further development.

While it is generally appreciated that mechanistic work and the development of meaningful SAR's are hampered by problems of a biological nature (e.g. the state of knowledge of the etiology of cancer, the limitations of present techniques in cell biology, the

influence of screening methodology on outcome, the variable response of different screens to compound structure, etc.), it is less frequently recognized that both areas also are affected by chemical problems, some of which are unique to metal compounds.

First, it is unfortunate that in many cases neither the purity or structures of new compounds have been verified by presently available techniques (HPLC, NMR, or, when possible, single crystal XRD) prior to their evaluation in antitumor screens. This can be particularly troublesome when the starting material for a preparation is cisplatin or another active diamine complex or the diaqua species derived from these. Second, an insufficient amount of attention is frequently paid to reactions which can occur in solution or when compounds are introduced into cell growth media. The latter becomes particularly important with the increased use of cell culture screening. It must be kept in mind that platinum compounds, even when relatively inert in aqueous solution may not be as stable in phosphate buffered saline at 37°C and likely will react at some measurable rate with fetal calf serum and other common culture additives. The ubiquitous practice of dissolving sparingly soluble compounds in DMSO which complexes readily with platinum, can and undoubtedly has resulted in potentially misleading results both in screening and mechanistic studies (24). These problems are aggrevated when stock solutions containing reactive compounds are employed.

Despite the problems encountered, the prospects for discovering new platinum compounds which are not only less toxic but also are active against presently resistant tumors remain good. Based on the small number of complexes that have been clinically evaluated so far, it should not be assumed that all platinum complexes will display activities similar to those displayed by cisplatin. There is, however a need to be innovative. In the absence of predictive screens, subjective judgements are likely to determine which compounds will be developed. Simple analogs of cisplatin, or those perceived to be, are unlikely to be selected. Compounds with novel structures, those which can be expected to display unique chemistry, or those designed in accordance with a specific rationale are more

likely to receive serious attention. There is at least some indication that antitumor activity is not limited to compounds conforming to established SAR's. Even among those which do, new ligand systems may modify the chemistry of the complexes sufficiently to alter selectivity. Other new approaches which are just beginning to be explored include complexes which incorporate ligands targeted to particular cellular receptors (25) or conjugates with monoclonal antibodies as well as new methods of drug delivery such as encapsulation in liposomes (26). The achievement of optimum effectiveness in the latter case also will undoubtedly require development of new complexes.

The rational design of new structures obviously will be greatly aided by an improved understanding of tumor cell biology, the development of more predictive screens and a more detailed knowledge of the mechanism of action of cisplatin.

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THE ANTICANCER ACTIVITY OF METAL PHOSPHINE COMPLEXES

S.J. Berners Price and P.J. Sadler

INTRODUCTION

Phosphines

Phosphines are the analogues of amines in nitrogen chemistry. However, unlike amines, phosphines never appear to take part in natural mammalian biochemistry. This is all concerned with oxidised phosphorus, P(V): phosphates and phosphate esters.

The simplest phosphine is PH_3, phosphine itself. It is a very different molecule to NH_3. With a pK_a of -14, it would not be protonated at biological pH (7.4), whereas NH_3 is: NH_4^+, pK_a 9.2. PH_3 is a very strong reducing agent, being readily oxidized to phosphate. The formation of such non-toxic decomposition products favours its use in the control of insect pests in stored agricultural goods (1). PH_3 inhibits mitochondrial respiration. This may involve direct binding to iron in haems.

Tertiary phosphines

This article is concerned with organo-substituted tertiary phosphines PR_3, bridged tertiary diphosphines $R_2P(CH_2)_nPR_2$, related compounds and their metal complexes. All phosphines have a lone pair of electrons on phosphorus. They can behave as bases and nucleophiles. The substituents R have a large effect on proton affinities: phenyl phosphines are weakly basic (pK_a ca. 3) and would not be protonated at pH 7, whereas some alkyl phosphines would be protonated (the pK_a of PEt_3 is 8.7).

The greater size and lower electronegativity of P compared to N leads to higher polarizability and higher nucleophilic reactivity for P. Phosphines are more effective in stabilizing low oxidation states of metal ions, e.g. Cu(I), Au(I) and Rh(I).

This is often attributed to the presence of empty 3d orbitals on P so that d-π back-bonding with filled metal orbitals can occur. Phosphines are π-acceptor ligands. The driving force for many chemical reactions of organophosphines is the formation of the very stable phosphoryl bond in the oxidized product $R_3P{=}O$.

ANTITUMOUR ACTIVITY

Early tests

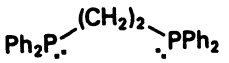
In 1966 Struck and Shealy (2) isolated 1,2-bis(diphenylphosphino)ethane (dppe) as a by-product from the synthesis of $Ph_2P(CH_2)_2Cl$, a nitrogen mustard analogue. It was reported to be cytotoxic to Eagles KB cells in vitro at concentrations 0.25 μM. Its antitumour activity was evaluated in 3 models: Sarcoma 180, active at 0.63 mmol (250 mg)/kg/day but not reproducible; adenocarcinoma 755, marginal activity at 1 mmol/kg/day; and L1210 leukaemia, inactive.

Recent re-investigations of the cytotoxicity and antitumour activity of dppe by Mirabelli et al. (3) have demonstrated activity against a wide range of tumours at much lower doses (40 μmol/kg). It is possible that dppe had become partially oxidized in the earlier studies, either before or during the tests. Alkyl phosphines are even more susceptible to air oxidation (4) and this has to be remembered when attempts are made to obtain reliable biological activity data.

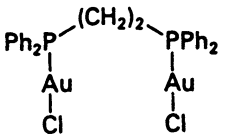
Cytotoxicity of auranofin

Current interest in the antitumour activity of phosphines arises from the observation by Lorber and coworkers (5) that the orally-active antiarthritic drug auranofin (SK & F 'Ridaura', for structures see Fig. 1) is potently cytotoxic toward cultured HeLa cells. Auranofin contains Au(I) linearly coordinated to PEt_3 and tetraacetyl-β-D-thioglucose. They also reported (5) that auranofin given i.p. increased the survival times of mice with i.p. P388 leukaemia.

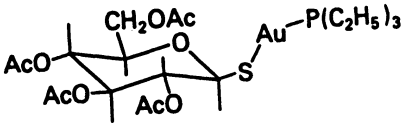
However, in subsequent detailed investigations (6) auranofin was found to exhibit activity in only one of 15 mouse tumour models i.p. P388 leukaemia, and then only if given i.p. In this series,



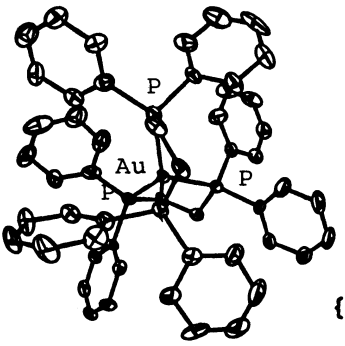
dppe

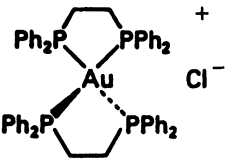


ClAu(dppe)AuCl

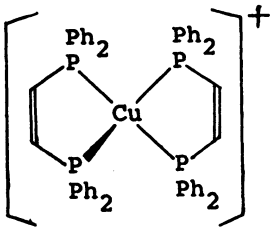


Auranofin

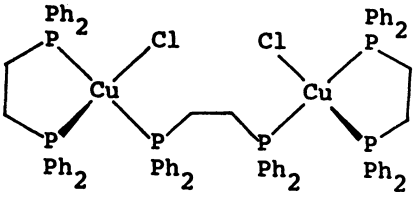




{Au(dppe)$_2$}$^+$



{Cu(vpp)$_2$}$^+$



{(CuCl)$_2$(dppe)$_3$}

Fig. 1. The structures of some of the phosphines and metal phosphine complexes discussed in this article.

activity is lost when PEt_3 is replaced by $PEtPh_2$ or PPh_3, although other active PPh_3 complexes of Au(I) have been reported (7-9).

Auranofin is potently cytotoxic towards a variety of tumour cells in vitro. The concentration required to reduce the number of colonies of B16 melanoma cells by 50% following a 2 hr exposure, IC_{50}, is 1.5 μM (6). It does not alter cell cycle distribution nor preferentially kill cells in logarithmic growth. DNA, RNA and protein syntheses are inhibited non-selectively at concentrations which are acutely lethal to cells. The cellular response is rapid and cells treated with 1 μM auranofin for 2 hr exhibit extensive morphological changes including membrane blebbing, cell rounding and membrane pitting. Related Au(I) complexes which do not contain the phosphine are usually not cytoxic nor antitumour-active (11).

The lack of a broader spectrum of antitumour activity for auranofin may be related to its interaction with plasma proteins. It reacts with albumin via thiol exchange at Cys 34 (10). The presence of serum reduces its in vitro cytotoxicity by a factor of 10 (6). Such reactions not only compete with uptake but also facilitate displacment of PEt_3 and its oxidation to $OPEt_3$.

Au(I) binds weakly to DNA. Gold(I) is an unstable oxidation state of gold unless bound to π-acceptor ligands such as thiolates or phosphines. Gold(I)-N bonds are much more labile than Pt(II)-N-bonds. Although Et_3PAuCl binds to plasmid DNA, auranofin does not (12): N ligands of DNA can displace Cl^- but not RS^-. Gold(III) is isoelectronic with Pt(II), $5d^8$, also forms square-planar complexes and binds more strongly to N ligands. A few Au(III) complexes such as $[Au(CH_3)_2Cl_2]^-$ are active (8), but in general they are too readily reduced to Au(I). The disphosphine complex $Cl_3Au(dppe)AuCl_3$ is active against P388 leukaemia (3, 13).

Diphosphine complexes

Complexation of $Ph_2PCH_2CH_2PPh_2$(dppe) with Au(I) protects the diphosphine from air oxidation. The bridged complex ClAu(dppe)AuCl is more cytoxic than dppe alone: IC_{50}'s for B16 melanoma cells are 8 μM and 60 μM respectively (13). The activity of the complex against i.p. P388 leukaemia (98% ILS) approaches that of cisplatin

(125% ILS) at similar MTD's (7 μmol/kg). Moreover the bridged complex, unlike auranofin, is active after i.p. administration against a range of tumours: L1210 leukaemia, M5076 reticulum cell sarcoma, B16 melanoma, mammary adenocarcinoma 16/c (i.p. tumours) and ADJ-PC6 plastocytoma (s.c. tumours) (3).

Structure-activity relationships for these bridged digold complexes have been investigated (3, 13):

1. The phosphine is important for activity. Replacement of P with As or S leads to inactivity, even though the complexes remain cytotoxic.
2. Activity is greatly reduced when the bridge contains less or more than 2 carbons.
3. When the bridge is rigid, the stereochemical configuration is important; cisCH=CH is active but trans CH=CH is inactive as are -C≡C- and 1,4-phenyl bridges (Ph substituents on P).
4. Activity is retained when Ph substituents on P are replaced by C_6D_5, cyclohexyl or m- or p-F-phenyl, but lost with benzyl, tolyl, ethyl or thienyl substituents (CH_2CH_2 bridge).

The diphosphine ligands themselves exhibit a very similar structure-activity series, Fig. 1. The bis-oxide $Ph_2P(O)CH_2CH_2P(O)Ph_2$ is inactive. This suggests that although gold may play a role, it is the diphosphine which is the major determinant of activity. Peaking of activity (Fig. 2) occurs for those ligands which could form chelated metal complexes, e.g. $(CH_2)_n$ with n = 2 or 3 or cis CH=CH, giving 5 or 6-membered rings.

Indeed, the cytoxic potency of dppe is increased when incubated with Au(III) and Cu(II) salts (12). A dppe concentration of 25 μM produces a 15% reduction in colony formation by B16 cells; this increases to 65% and 85% in the presence of 10 μM and 50 μM added $CuCl_2$ respectively. Mg(II), Zn(II), Mn(II), Fe(II), Co(II) and Cd(II) are not effective. Both Au(III) and Cu(II) are likely to be reduced by dppe to Au(I) and Cu(I) which may then complex to dppe, perhaps via chelation (14).

The formation of chelated complexes may play a role in the molecular pharmacology of XAu(dppe)AuX compounds. When X=thioglucose, conversion into $[Au(dppe)_2]^+$ can be observed in

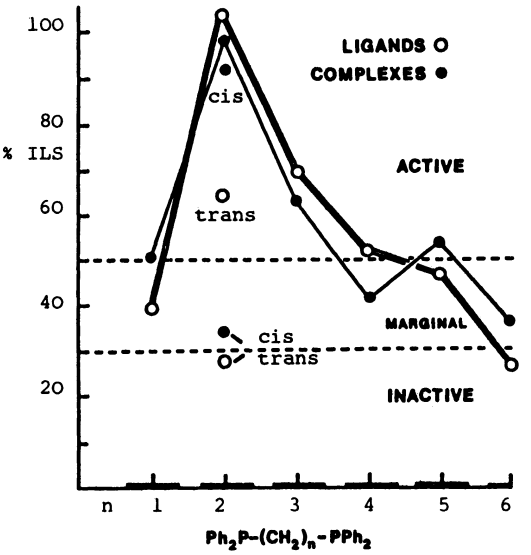


Fig. 2. Dependence of the activity of diphosphines and bridged complexes $ClAu(Ph_2P(CH_2)_nPPh_2)AuCl$ on the length of the bridge n: the points labelled cis and trans refer to CH=CH bridges. Data from ref. 3.

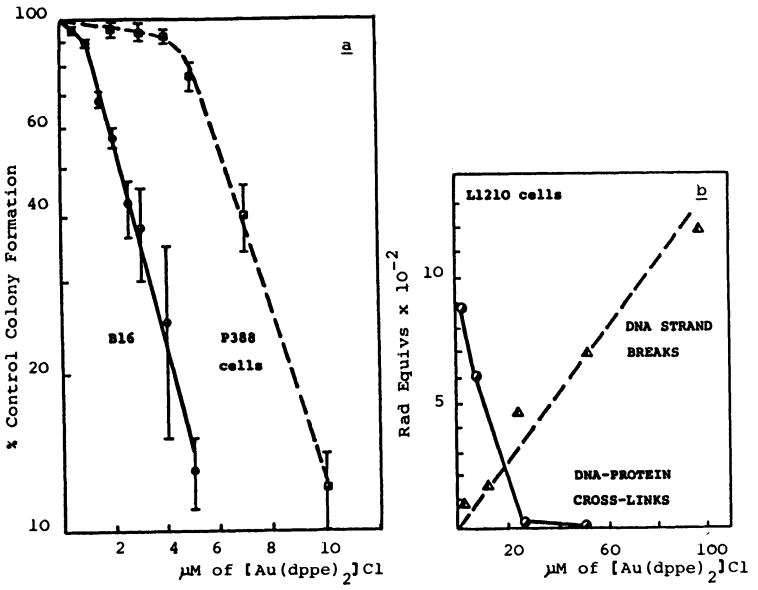


Fig. 3 a) Cytotoxic activity of $[Au(dppe)_2]Cl$ against B16 and P388 cells in colony formation assays following 2-h exposures. b) DNA strand breaks and DNA-protein cross-links in L1210 cells after 1 hr treatment, 37° (adapted from ref. 19).

blood plasma *in vitro* by ^{31}P NMR (15). Reaction of glutathione with the X=Cl complex also follows the same path.

TETRAHEDRAL BIS(DIPHOSPHINE) COMPLEXES

The chemistry of four-coordinate, bischelated Au(I) diphosphine complexes has been little studied and their biological properties have not been previously investigated.

Chelation of diphosphines such as $Ph_2P(CH_2)_nPPh_2$ with n = 2,3 to Au(I) in solution can be observed by ^{31}P NMR (16, 17). Stable complexes can be isolated and characterized. The $[Au(dppe)_2]^+$ ion has a flattened tetrahedral geometry (16, 18), see Fig. 1. Unlike the two-coordinate Au(I) complexes discussed above, it does not readily react with thiols (19). When added at millimolar levels to blood *in vitro* ^{31}P NMR studies suggest that it is about equally distributed between the plasma and red cell membranes (20).

Antitumour activity of $[Au(dppe)_2Cl$

At a maximum tolerated dose (MTD) of 2.9 μmol/kg (i.p. daily, 5 d), $[Au(dppe)_2]Cl$ produced an average 87% ILS for mice bearing P388 leukaemia over 21 separate experiments: the tumour cell burden was reduced by 99% by the end of treatment (19). The complex was less toxic when given i.v., s.c., or p.o., but was inactive in this tumour system by these routes of administration. It was also inactive against i.v. innoculated P388 leukaemia when given i.p. or i.v.

$[Au(dppe)_2]Cl$ was active against i.p. implanted M5076 reticulum cell sarcoma, producing a 59% ILS at an MTD of 1.9 μmol/kg/d (i.p. daily, 10 d). As with P388 leukaemia, there was a dose-dependent prolongation of lifespan (19). The complex was also active against B16 melanoma (39% ILS), the s.c. implanted tumour mammary adenocarcinoma 16/c when administered either i.p. or i.v.

$[Au(dppe)_2]Cl$ was found to be active against both a parental and cisplatin-resistant P388 tumours. Combinations of cisplatin and $[Au(dppe)_2]Cl$ were more effective than the individual complexes alone in prolonging the lifespan of moderately advanced tumour-

bearing mice (19). This suggests that the mechanism, or site, of action is different from cisplatin.

Activity of analogues. The mixed substituent complex $[Au(Et_2PCH_2CH_2PPh_2)_2]Cl$ is slightly less active against P388 leukaemia than the complex containing only Ph substituents. $[Au(Et_2PCH_2CH_2PEt_2)_2]PF_6$ is inactive, paralleled by an almost 4-fold lower toxicity (clonogenic assay) toward B16 cells (20).

It is possible to substitute Au(I) for the other group IB metals Cu(I) or Ag(I) and retain activity, e.g. $(CuCl)_2(dppe)_3$ (see Fig. 1) and $[Ag(dppe)_2]Cl$ are active (20). Although the chelate rings present in these complexes are thermodynamically stable, the metal-P bonds are kinetically labile and reactions can occur via ring-opening mechanisms (21, 22).

The metal ion may protect the diphosphine from oxidation and deliver it to intracellular target sites. Complexes such as $M(dppe)_2Cl_2$, M = Pd(II) or Pt(II) are inactive (3), perhaps because of insufficient M-P bond lability.

Cytotoxicity

$[Au(dppe)_2]Cl$ is cytotoxic to tumour cells in vitro at micromolar concentrations. The survival curves are characterised by a shoulder at low concentrations with exponential cell kill at higher concentrations, Fig. 3 (19). This behaviour is similar to that for radiomimetric agents. The IC_{50} values for B16 and P388 cells (cologenic assay, 2 hr exposure) are 2 μM and 6 μM respectively, and only 2-fold lower in a medium not containing serum. The concentration of the complex required to produce acute cell death is at least 20 x that required to inhibit the clonogenic capacity of B16 cells. This is in contrast to auranofin for which cytotoxic effects appear to be acutely mediated (immediate) (6), accompanied by monophasic exponential survival curves.

How does $[Au(dppe)_2]Cl$ interfere with the reproductive capacity of cells? It has been found to inhibit the biosynthesis of DNA, RNA and proteins and to produce a concentration-dependent increase in DNA single strand breaks which are not removed on drug removal as measured by the alkaline elution technique for L1210 cells (19). At low concentrations of complex (<5 μM), DNA-protein cross-

links appeared to be more significant, Fig. 3.

A role for copper?

[Au(dppe)]Cl reacts with Cu(II) ions to give a Cu(I) complex (19), and the activity of some diphosphines is potentiated by Cu(II) (13). It is possible that copper plays an important role in the mechanism of action of this class of compounds, as suggested for some other cytotoxins such as 2,9-dimethyl-1,10-phenanthroline and thiosemicarbazones (see ref. 13).

$(CuCl)_2(dppe)_3$, $[Cu(vpp)_2]Cl$, $[Au(dppe)_2]Cl$ (see Fig. 1) and dppe itself all produce DNA-protein cross-links in cultured mammalian cells at biologically-relevant concentrations (0.1 μM) (23). Cells rendered resistant to dppe (12x) are also resistant to the Cu(I) and Au(I) complexes (5-7x). This suggests that the compounds act through a common mechanism.

Conclusion

Certain metal phosphine complexes have now been shown to have significant and reproducible antitumour activity in a broad spectrum of murine tumour models and potent cytotoxic activity to tumour cells in vitro. In some cases the phosphine lignads alone are active but they are often more susceptible to oxidation, less potent, less soluble in aqueous media and difficult to formulate. The mechanism of action of metal diphosphine complexes appears to be different from that of cisplatin.

Further development of these complexes will depend on the results of detailed toxicity testing now in progress. There is much scope for the study of phosphine chemistry and biochemistry under biologically relevant conditions (24). This may lead to improved design of this class of cytotoxic agents.

Acknowledgements

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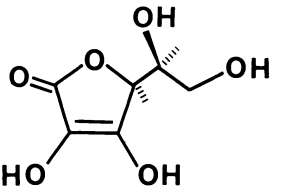
PLATINUM-BASED ORGANOMETALLIC COMPLEXES OF VITAMIN C AND RELATED ANALOGS AS ANTITUMOR AGENTS

S. L. Hollis, S.L. Doran, A.R. Amundsen and E.W. Stern

Introduction

While cisplatin has proven effective as a antitumor agent in treating a variety of forms of cancer, such as testicular and ovarian carcinomas,[1] a substantial effort has been made in new drug development programs to improve the therapeutic properties of platinum-based antitumor agents.[2] Major objectives of research in this area include the development of new agents that have an improved spectrum of activity and are more effective and less toxic than cisplatin.[3] To date these efforts have produced a number of new compounds that show good activity in various animal tumor screens.[4] While recent clinical studies show that some second-generation analogs, such as carboplatin, cis-[$Pt(NH_3)_2$-(1,1 -cyclobutanedicarboxylate)][5], are less toxic than cisplatin, the search for new agents that display a significantly different spectrum of activity is continuing in laboratories worldwide.

Recently we have been studying the chemical and biological properties of a new class of antitumor agents based on cis-diamineplatinum(II) complexes of ascorbic acid. These compounds are prepared by reacting sodium ascorbate with a variety of diaquadiamine complexes of the form cis-$[Pt(RNH_2)_2(H_2O)_2]^{2+}$. Multinuclear NMR studies of these reactions show that the ascorbate anion is a multifunctional ligand that is capable of binding to platinum at a number of sites (O2, O3, C2, and O5), in either a monodentate or bidentate fashion.



Ascorbic Acid (H_2Asc)

Solution Studies of the cis-Diamineplatinum(II)-Ascorbate Complexes

^{195}Pt NMR spectroscopy was used to identify the various products of the diamineplatinum-ascorbate reactions. The high receptivity of the ^{195}Pt nucleus, the broad chemical shift range, and the predictable nature of the ^{195}Pt chemical shifts upon ligand substitution provide an excellent means for product identification. Additional structural information is provided by the ^{195}Pt-^{15}N coupling constant data that can be obtained by using ^{15}N-labeled starting materials. Since the kinetics of these reactions are relatively slow, this technique also proves to be a convenient method for monitoring product distributions as a function of time.

Previous NMR studies have shown that the magnitude of the coupling constant, $^{1}J(^{195}Pt\text{-}^{15}N)$, is related to the donor properties of the ligands bound to platinum.[6] In general, the X and Y ligands in complexes of the form cis-$[Pt(^{15}NH_2R)_2(X)(Y)]$ exert both cis- and trans-influences on the ^{195}Pt-^{15}N coupling constants. While there are a number of factors that are involved in this interaction,[6] the trans-influence typically dominates the relationship between the ligand donor strength and the ^{195}Pt-^{15}N coupling constant. Typically, the coupling constants are smaller when the ^{15}N-labeled amine is trans to a strong donor such as sulfur or a carbon-based ligand than when it is trans to a weak donor such as water or an oxygen-based ligand. The relationship

between ligand donor strength and the ^{195}Pt-^{15}N coupling constants is easily demonstrated by plotting the ^{195}Pt chemical shift, which is a measure of ligand donor strength, versus the magnitude of ^{1}J for a series of related platinum complexes. As shown in Figure 1,

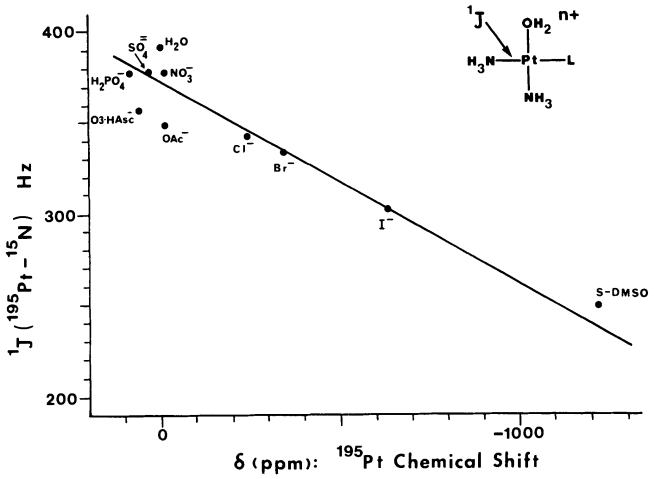


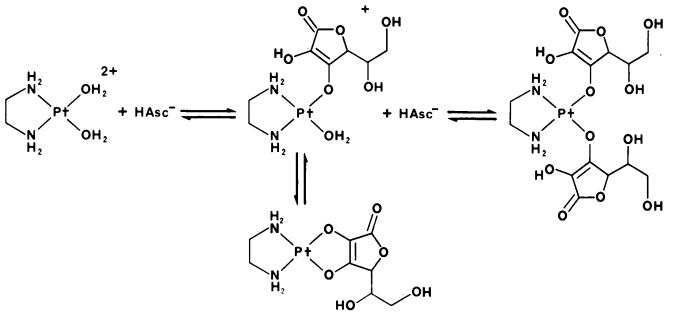
Figure 1. Plot of ^{195}Pt chemical shift as a function of the ^{195}Pt-^{15}N coupling constant trans to various L-donors for a series of monoaqua complexes: cis-$[Pt(^{15}NH_3)_2(H_2O)(L)]^{+/o}$. The chemical shift axis is referenced relative to cis-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ at 0 ppm for illustrative purposes. The data presented in this plot are taken from references 6 and 7.

the NMR data obtained on a series of monoaqua complexes of the form cis-$[Pt(^{15}NH_3)_2(H_2O)(L)]^{+/o}$ indicate that the magnitude of the coupling constant trans to various L donors correlates well with the donor strength of L. This relationship was used as an aid in the identification of the various products of the diamine-platinum ascorbate reactions.[7]

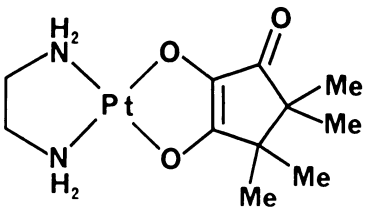
^{195}Pt NMR studies of the reaction between aqueous sodium ascorbate and $[Pt(^{15}en)(H_2O)_2]^{2+}$ (1) show that two complexes of the ascorbate monoanion ($HAsc^-$) and one complex of the ascorbate dianion (Asc^{2-}) are produced during the initial stage of the reaction ($t < 1$ hr). These oxygen-bound species are the mono-

ascorbate complex [Pt(^{15}en)(O3-HAsc)(H_2O)]$^+$ (2), the bisascorbate complex [Pt(^{15}en)(O3-HAsc)$_2$] (3), and the ascorbate chelate [Pt(^{15}en)(O2,O3-Asc)] (4). The ^{195}Pt chemical shift and ^{195}Pt-^{15}N coupling constant data for the various products of this reaction are presented in Table 1 and the reaction sequence involving these species is depicted in Scheme I. From the coupling constant data

Scheme I



obtained on the two O3-bound complexes,[7] the ascorbate ligand was found to be a better donor than water, as $^1J(^{195}Pt-^{15}N)$ decreases from 420 Hz in the diaqua complex (1) to 385-390 Hz in 2 and 3. As expected, based on the additive nature of substitution shifts in ^{195}Pt NMR, a relatively constant change in chemical shift per ascorbate substitution was found in this series (~+65 ppm per $HAsc^-$, in going from 1 to 2 to 3). The third oxygen-bound complex was identified as the O2,O3-ascorbate chelate 4 based on the coupling constant and chemical shift data and by comparison to a model compound of a related enediol chelate, [Pt(^{15}en)(O2,O3-



[Pt(en)(O2,O3-TMRA)]

Table 1. ^{195}Pt NMR Data, Chemical Shifts (ppm) and ^{195}Pt-^{15}N Coupling Constants (Hz) for 'Pt(^{15}en)(ascorbate)' Complexes.

Compound	Chemical Shift[a]	J	J'
Pt(^{15}en)(ascorbate) Reaction Products			
[Pt(^{15}en)(H_2O)$_2$]$^{2+}$ (1)	-1911	420	
[Pt(^{15}en)(H_2O)(O3-HAsc)]$^{+}$ (2)	-1838	422[b]	385[c]
[Pt(^{15}en)(O3-HAsc)$_2$] (3)	-1780	390	
[Pt(^{15}en)(O2,O3-Asc)] (4)	-1712	363	
[Pt(^{15}en)(O2,O3-TMRA)] (6)	-1806	360	
[Pt(^{15}en)(C2,O5-Asc)] (7)	-2634	347[d]	253[e]
[Pt(^{15}en)(C2-HAsc)(O3-HAsc)] (8)	-2663	400[c]	237[e]
Ring-Opening Reaction Products			
[Pt(^{15}en)(C2,O5-HAsc)]$^{+}$ (9)	-2706	442[d]	244[e]
[Pt(^{15}en)(C2-HAsc)(H_2O)]$^{+}$ (10)	-2810	450[b]	222[e]
[Pt(^{15}en)(C2-HAsc)Cl] (11)	-3105	404[f]	211[e]
[Pt(^{15}en)(C2-HAsc)(S-DMSO)]$^{+}$ (12)	-3660	296[g]	209[e]
[Pt(^{15}en)(O3-HAsc)(S-DMSO)]$^{+}$ (14)	-2929	376[c]	298[g]
[Pt(^{15}en)(H_2O)Cl]$^{+}$	-2122	403[b]	381[f]
[Pt(^{15}en)(H_2O)(S-DMSO)]$^{2+}$	-3002	409[b]	293[g]

[a] Chemical shifts measured relative to H_2PtCl_6 (1 g in 3 mL D_2O) at 0 ppm. [b] trans to H_2O. [c] trans to O3. [d] trans to O5. [e] trans to C2. [f] trans to Cl. [g] trans to S.

tetramethylreductate)] (**6**). Tetramethylreductic acid (TMRA) was chosen as an ascorbic acid model because the redox properties and the enediol functionality of this molecule are similar to those of vitamin C.[7]

The kinetically favored oxygen-bound species, which predominate in the early stages of the reaction, convert to two carbon bound complexes as the reaction proceeds. These products are the monoascorbate complex, [Pt(^{15}en)(C2,O5-Asc)] (**7**), which contains a dianionic ascorbate ligand bound through the C2-carbon atom and a deprotonated hydroxyl group (O5), and the bisascorbate complex, [Pt(^{15}en)(C2-HAsc)(O3-HAsc)] (**8**), which contains both C2- and O3-bound ascorbate ligands. The C2-ascorbate ligand in both the C2,O5-chelate (**7**) and the bisascorbate complex (**8**) can be viewed as an α-hydroxy-ß-diketonate ligand which is attached to platinum on the rectus (re) or sinister (si) face of the ascorbate ring, respectively. ^{195}Pt NMR studies of these products (see Table 1) indicate that the C2-ascorbate ligand in both complexes is a strong donor; ^{1}J trans to C2 is in the 230 to 260 Hz range. Additional NMR (^{195}Pt and ^{13}C) and X-ray crystallographic studies have been conducted on the carbon-bound products of the diamineplatinum-ascorbate reactions, where the diamine ligand is R,R-, S,S- or R,S-diaminocyclohexane (dach), or $(NH_3)_2$. In each of these cases, the carbon-bound complexes (si and re) form in a stereoselective fashion as the final products of the reaction. The preferred carbon binding site was found to lie on the re face of the ascorbate ring. When platinum binds to this site, the exocyclic glycol chain of the ascorbate ligand is oriented in a position that favors chelate formation. As a result, the O5-hydroxyl group binds to platinum with loss of a proton, yielding the C2,O5-ascorbate chelate. These products are typically isolated as crystalline precipitates in yields up to 65%. Two of the chelates, [Pt(R,S-dach)(C2,O5-Asc)][8] and [Pt(S,S-dach)(C2,O5-Asc)][9], have been characterized by using single crystal X-ray diffraction techniques (see Figure 2).

When platinum binds to the si face of the ascorbate ligand at the C2 carbon, the exocyclic glycol chain is positioned away from

the metal on the opposite side of the lactone ring. Since ring closure is prohibited in this case, the remaining binding site on the platinum complex is available for further reaction with a second ascorbate anion when excess sodium ascorbate is present in

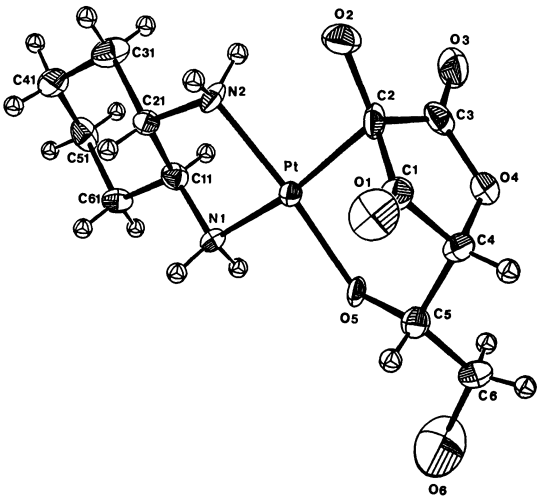
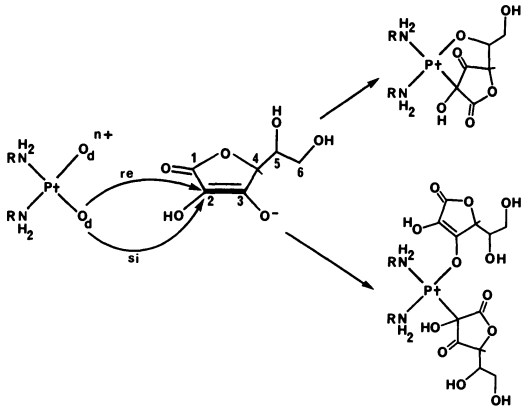


Figure 2. ORTEP illustration of the structure of the [Pt(S,S-dach)(C2,O5-Asc)] chelate, showing the 40% probability thermal ellipsoids for all non-hydrogen atoms.

solution. As a result, bisascorbate complexes form under these conditions (see Scheme II). While ^{195}Pt NMR studies indicate that both carbon-bound ascorbate complexes form at similar rates, the

Scheme II



chelate to bisascorbate (re:si) ratio in the product was found to vary, in the range of 1.5 to 3.3, as a function of the particular diamine ligand used in the reaction. These results show that the reaction is stereoselective for the face of the ascorbate ring that is able to bind to platinum in a bidentate fashion.

In general, for each of the diamineplatinum ascorbate reactions that have been studied by NMR, the products are analogous to those described in Schemes I and II. One major difference was found, however, when the reaction was run with the diammine complex, cis-$[Pt(^{15}NH_3)_2(H_2O)]^{2+}$. In this case, additional products are formed as a result of ammonia release at the site trans to the C2-ascorbate ligand. These products are monoamine complexes of the form $[Pt(^{15}NH_3)(O_d)(C2,O5\text{-Asc})]^{+/o}$, where O_d is H_2O or O3-$HAsc^-$. Exchange occurs at this site as a result of the strong trans-influence of the C2-donor. SCF calculations, which were performed on cis-$[Pt(NH_3)_2(C2,O5\text{-Asc})]$,[10] also suggest that exchange is likely to occur at the site trans to C2, based on the calculated Pt-N bond energies (15 kcal/mol trans to C2 and 36 kcal/mol trans to O5).

Reaction Chemistry of the cis-Diamineplatinum-Ascorbate Chelates

While antitumor activity has been found for both the ascorbate chelates and the bisascorbate complexes in animal tumor screens, the ascorbate chelates are generally less active than the bisascorbate analogs (see Table 2). Since the C2,O5-ascorbate chelates are the major products of these reactions, efforts were made to convert the chelates to ring-opened analogs of the bisascorbate complexes: cis-$[Pt(diamine)(C2\text{-HAsc})(X)]^{+/o}$, where X is a neutral or anionic ligand.[11] Since chelate formation through the C2 and O5 binding sites occurs with the loss of two protons, we chose to examine the protonation reactions of the chelate as a possible pathway to ring-opened complexes of this form.

A series of ^{195}Pt and ^{13}C NMR experiments was conducted to study the chelate protonation process. NMR studies of the titration of $[Pt(S,S\text{-dach})(C2,O5\text{-Asc})]$ with aqueous HNO_3 show that

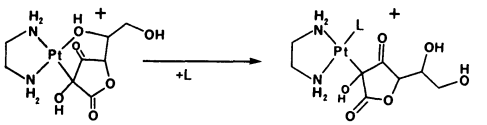
Table 2. Screening Results for cis-Diamineplatinum(II) Ascorbate and 1,3-Cyclicdione (CD) Complexes.

Compound[b]	S180a[a] Best %ILS	S180a[a] Dose (mg/kg)	L1210[a] Best %ILS	L1210[a] Dose (mg/kg)
[Pt(en)(C2,O5-Asc)]	24	320		
cis-[Pt(i-PrNH$_2$)$_2$(C2,O5-Asc)]	11	80		
cis-[Pt(NH$_3$)$_2$(C2,O5-Asc)]	79	320		
[Pt(R,R-dach)(C2,O5-Asc)]	52	320		
[Pt(S,S-dach)(C2,O5-Asc)]	37	320		
[Pt(R,S-dach)(C2,O5-Asc)] isomer 1	103	80		
[Pt(R,S-dach)(C2,O5-Asc)] isomer 2	98	160		
[Pt(R,S-dach)(C2-HAsc)(O3-HAsc)]	97	160		
[Pt(en)(C2-CHD)(O1-CHD)]	90	160	10	160
cis-[Pt(i-PrNH$_2$)$_2$(C2-CHD)(O1-CHD)]	17	10		
cis-[Pt(NH$_3$)$_2$(C2-CHD)(O1-CHD)]	45	160		
[Pt(R,R-dach)(C2-CHD)(O1-CHD)]	98	160	155	320
[Pt(S,S-dach)(C2-CHD)(O1-CHD)]	71	320	118	160
[Pt(R,S-dach)(C2-CHD)(O1-CHD)]	10	320	20	320
[Pt(R,R-dach)(C2-MCHD)(O1-MCHD)]	91	80	103	160
[Pt(R,S-dach)(C2-MCHD)(O1-MCHD)]	1	160	-7	160
[Pt(R,R-dach)(C2-DCHD)(O1-DCHD)]	-6	40		
[Pt(R,R-dach)(C2-CPD)(O1-CPD)]	98	160		
[Pt(en)(C3,O8-CPD$_2$)]	10	40		
Cisplatin[c]	81(12)	8	87(39)	8

[a] Test protocols are given in ref 15. [b] Abbreviations are given in ref 19. [c] Data given are average (std dev) for positive control (8 mg/kg cisplatin administered in 0.15 M saline).

the ^{195}Pt resonance shifts upfield by 70 ppm in a reversible fashion, as a result of protonation. The pK_a of the protonated complex was determined to be 2.0 from the ^{195}Pt NMR titration data.[11] Additional ^{195}Pt and ^{13}C NMR studies of the reaction of [Pt(^{15}en)(C2,O5-Asc)] (**7**) with HNO_3 in water indicate that protonation occurs at the O5 site of the ascorbate ligand.[11] In the absence of a nucleophile (X), the protonated form of the chelate cis-[Pt(^{15}en)(C2,O5-HAsc)]$^+$ (**9**), is favored over the ring-opened complex, cis-[Pt(^{15}en)(C2-HAsc)(H_2O)]$^+$ (**10**). While the protonated complexes can be prepared (using CH_3SO_3H) and isolated from non-aqueous solvents (such as methanol, ethanol, or DMF), they were found to decompose to ascorbic acid and the starting diaqua complex, cis-[Pt(diamine)(H_2O)$_2$]$^{2+}$, upon standing in aqueous solution.

When the protonation reactions were conducted in aprotic solvents in the presence of a nucleophile, such as Cl^- or DMSO, ring-opened complexes were isolated. These compounds were characterized by using ^{13}C and ^{195}Pt NMR (see Table 1). For example,



Solvent:
(1) ROH
(2) DMF
(3) DMSO

the reaction of [Pt(^{15}en)(C2,O5-Asc)] with CH_3SO_3H/LiCl in dry DMF (equation 1) produces the ring-opened chloroascorbate complex **11**. Analogous ring-opened complexes containing a sulfur-bound DMSO ligand can be prepared by reacting the C2,O5-chelate with CH_3SO_3H in DMSO (equation 2). Both the chloro- and sulfur-bound complexes can be isolated from solution by precipitation with acetone and ether. HPLC and ^{195}Pt NMR studies show that, in dilute aqueous solution, the chloroascorbate complex rapidly undergoes ring-closure to the C2,O5-chelate. However, when the ring-opened DMSO

(1) $[Pt(^{15}en)(\underline{C2},\underline{O5}\text{-Asc})] + H^+ \xrightarrow{LiCl} [Pt(^{15}en)(\underline{C2}\text{-HAsc})Cl]$
7 11

(2) $[Pt(^{15}en)(\underline{C2},\underline{O5}\text{-Asc})] + H^+ \xrightarrow{DMSO} [Pt(^{15}en)(\underline{C2}\text{-HAsc})(\underline{S}\text{-DMSO})]^+$
7 12

complex (12) is dissolved in water, the C2-bound ascorbate ligand isomerizes to the O3-bound product, $[Pt(^{15}en)(\underline{O3}\text{-HAsc})(\underline{S}\text{-DMSO})]^+$ (14). The dissociation of the C2-ascorbate ligand in 12 may result from the large cis-effect produced by the sulfur bound DMSO ligand.[12] In general, the results of these studies demonstrate that monodentate C2-bound ascorbate complexes are unstable relative to the C2,O5-chelate when platinum is bound to the re face of the ascorbate ring.

cis-Diamineplatinum(II) Complexes Containing Cyclic ß-Diketonate Ligands

Stable cis-diamineplatinum(II) complexes containing one monodentate carbon-bound ligand can be obtained when the analogous reactions are conducted by using ß-diketonate ligands that lack the ability to form a stable chelate ring. In this manner, a series of ß-diketonate complexes of the form cis-[Pt(diamine)(C2-CD)(O1-CD)], where CD is a cyclic ß-diketonate ligand, bound through the central carbon (C2) or through an enolate oxygen (O1), was prepared and characterized by using multinuclear NMR and X-ray crystallography. The reaction of $[Pt(\underline{R},\underline{R}\text{-dach})(H_2O)_2]^{2+}$ with the sodium salt of 1,3-cyclohexanedione (CHD) was found to be analogous to the diamineplatinum-ascorbate reaction as judged by ^{195}Pt NMR. The mono- and bisoxygen-bound complexes of the CHD-enolate ligand, $[Pt(\underline{R},\underline{R}\text{-dach})(\underline{O1}\text{-CHD})(H_2O)]^+$ and $[Pt(\underline{R},\underline{R}\text{-dach})(\underline{O1}\text{-CHD})_2]$, form in the early stage of the reaction and rapidly convert to carbon-bound products within 1 hr. The major carbon-bound species has been identified, with the aid of X-ray crystallography, as a direct analog of the carbon-bound bisascorbate complex (see Scheme II). The structure of this complex, $[Pt(\underline{R},\underline{R}\text{-dach})(\underline{C2}\text{-CHD})(\underline{O1}\text{-}$

CHD)] (**16**)[13], is depicted in the ORTEP illustration shown in Figure 3. As indicated from these studies, one CHD ligand is bound to platinum through a deprotonated carbon and the second ligand is attached as an enolate, through oxygen-O1. This analog of the bisascorbate complex, which is stable in aqueous solution, is formed as the major product of the reaction and is readily isolated and purified by using standard recrystallization techniques.

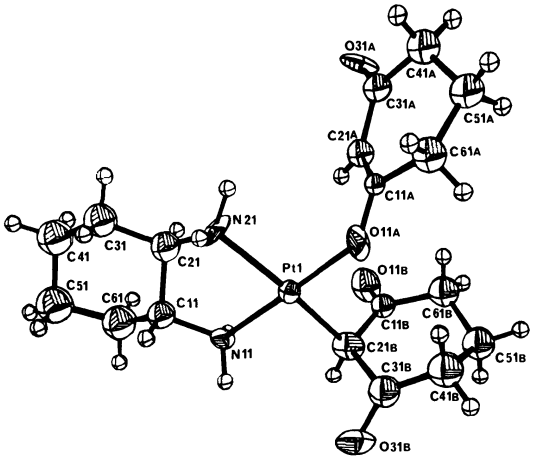


Figure 3. ORTEP illustration of the structure of the bis-CHD complex, [Pt(R,R-dach)(C2-CHD)(O1-CHD)] (**16**), showing the 40% probability thermal ellipsoids for all non-hydrogen atoms.

The results of ^{195}Pt NMR studies of this reaction, using the ^{15}en substituted diaqua complex $[Pt(^{15}en)(H_2O)_2]^{2+}$, are closely related to those found in the ascorbate system (see Table 3). The ^{195}Pt chemical shift and coupling constant data obtained on the bis-CHD analog, [Pt(^{15}en)(C2-CHD)(O1-CHD)] (**15**), compare favorably to those observed for the analogous bisascorbate complex (**8**). The ^{195}Pt-^{15}N coupling constant trans to the carbon donor in the bis-CHD complex (**15**) is approximately 30 Hz larger than that found in the bisacorbate analog (**8**). This increase apparently results from the enhanced donor strength of the hydroxylated C2-carbon in

Table 3. ^{195}Pt NMR Data, Chemical Shifts (ppm) and ^{195}Pt-^{15}N Coupling Constants (Hz) for 'Pt(^{15}en)(CD)' Complexes.

Compound	Chemical Shift[a]	J	J'
[Pt(^{15}en)(O1-CHD)(H_2O)]$^+$	-1871	408[b]	388[c]
[Pt(^{15}en)(O1-CHD)$_2$]	-1801	371[c]	
[Pt(^{15}en)(C2-CHD)(O1-CHD)] (15)	-2562	371[c]	264[d]
[Pt(^{15}en)(C2-CHD)(H_2O)]$^+$	-2629	429[b]	253[d]
[Pt(^{15}en)(C2-CHD)Cl]	-2904	397[e]	236[d]
[Pt(^{15}en)(C2-CPD)(O1-CPD)] (19)	-2533	369[c]	271[d]
[Pt(^{15}en)(C3,O8-CPD$_2$)] (20)	-2686	349[f]	149[g]

[a] Chemical shifts measured relative to H_2PtCl_6 (1 g in 3 mL D_2O) at 0 ppm. [b] trans to H_2O. [c] trans to O1. [d] trans to C2. [e] trans to Cl. [f] trans to O8. [g] trans to C3.

the ascorbate complex (**8**). As expected based on the coupling constant data, the weakly bound O1-CHD ligand can be displaced in proton-induced ligand exchange reactions. For example, the reaction of [Pt(R,R-dach)(C2-CHD)(O1-CHD)] (**16**) with aqueous HNO_3, followed by the addition of 1 equivalent of NaCl, produces the monochloro complex [Pt(R,R-dach)(C2-CHD)Cl] (**17**). In contrast to the monochloro-ascorbate complexes, the monochloro-CHD analogs are very stable in nonaqueous solvents. Furthermore, treatment of the chloro-CHD complex **17** with aqueous $AgNO_3$ produces the monoaqua complex [Pt(R,R-dach)(C2-CHD)(H_2O)](NO_3) (**18**), which is stable in aqueous solution. By reacting this product with various nucleophiles (X), a series mono-carbon bound complexes of the form cis-[Pt(diamine)(C2-CD)(X)] can be prepared.

Additional analogs of the bis-CHD complex (**16**) have been prepared and tested in the L1210 and S180a animal tumor screens

(see Table 2). Complexes containing one or two methyl groups substituted at the C5-position of the CHD ligand have been isolated and characterized by using the methods described above. Similarly, the related cyclopentane-1,3-dione (CPD) complex, [Pt(en)(C2-CPD)(O1-CPD)] (**19**), was obtained from the reaction of the sodium salt of CPD with $[Pt(en)(H_2O)_2]^{2+}$. In this case, however, a second product was isolated and characterized by X-ray crystallography[14]. As shown in Figure 4, this product, [Pt(en)-(C3,O8-CPD_2)] (**20**), contains a five-membered C,O-chelate ring that results from the dimerization of two CPD ligands. The CPD-dimer apparently forms as a result of the attack of the C2-carbon of one CPD anion on the carbonyl carbon on an adjacent CPD ligand, followed by elimination of water across the resulting carbon-carbon bond (C3-C7). Further studies of this reaction are in progress.

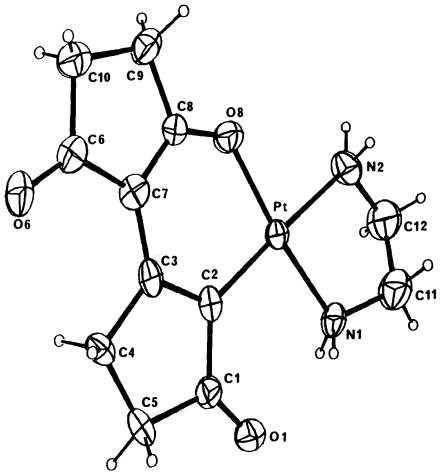


Figure 4. ORTEP illustration of the structure of the dimeric-CPD complex, [Pt(en)(C3,O8-CPD_2)] (**20**), showing the 40% probability thermal ellipsoids for all non-hydrogen atoms.

Antitumor Activity of cis-Diamineplatinum(II) Complexes Containing Carbon-Based Ligands

Antitumor activity of the carbon-bound ascorbate and ß-diketonate complexes was evaluated by using the S180a and L1210 murine tumor screens (Table 2). The data obtained on the ascorbic acid and the ß-diketonate complexes show that some members of this class have activity equal to or greater than cisplatin in one or both screens. The activity in the case of the cis-[Pt(diamine)-(C2,O5-Asc)] complexes was found only in the S180a screen, while the bisascorbate complexes displayed activity in S180a, L1210[18] and B16[18]. Representative ß-diketonate complexes, cis-[Pt(diamine)(C2-CD)(O1-CD)], demonstrate good activity in both S180a and L1210 screens.

The activity in this series of compounds appears to be influenced by the diamine ligands as well as the nature of the carbon-bound substituent. In the case of the C2,O5-ascorbate chelates, the best activity is found when the diamine ligand is R,S-dach (the cis isomer). The reverse effect is observed with the ß-diketonate complexes, where both R,R- and S,S-dach isomers show activity while the R,S-isomer does not. Variations of this sort, where the conformation of the diamine ligand is found to alter the observed antitumor activity, have been the subject of numerous studies.[20] In general, this effect appears to be a function of a number of factors, such as the nature of the leaving group, the solubility of the complex, and the choice of tumor model. A similar situation is found when the effect of the carbon-donor is examined. For example, the addition of one methyl group at the C5-position of the 5-methyl-CHD ligand (MCHD), in going from [Pt(R,R-dach)(C2-MCHD)(O1-MCHD)] to [Pt(R,R-dach)(C2-DCHD)(O1-DCHD)], results in a loss of activity in the S180a screen. Clearly, further studies will be required to develop a better understanding of the structure-activity relationships for this new series of antitumor agents.

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9. Crystallographic data. [Pt(S,S-dach)(C2,O5-Asc)]·$5H_2O$ crystallizes in the orthorhombic space group $P2_12_12_1$ with the following cell parameters: a = 12.366(1), b = 22.371(3), c = 6.782(1) Å, V = 1876.3 $Å^3$, Z = 4. The structure was solved by using standard Patterson and Fourier methods using 1943 reflections ($2\theta < 50^o$) collected on an Enraf-Nonius CAD-4F diffractometer. Refinement of the absorption corrected data, with all atoms (execpt H) assigned anisotropic thermal parameters converged at R_f = 0.033 and R_w = 0.045. Full details will be reported at a later date.
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13. Crystallographic data. [Pt(R,R-dach)(C2-CHD)(O1-CHD)] (**16**) crystallizes in the space group P1 with the following cell parameters: a = 10.225(1), b = 11.841(1), c = 9.387(1) Å, α= 112.15(1)°, ß = 106.15(1)°, γ= 87.91(1)°, V = 1008.2 $Å^3$, Z = 2. The structure was solved by using standard Patterson and Fourier methods using 4475 unique reflections ($2\theta < 54^o$). Refinement of the absorption corrected data, with all atoms, except Pt, N and O, assigned isotropic thermal parameters converged at R_f = 0.023 and R_w = 0.025. Full details will be reported at a later date.
14. Crystallographic data. [Pt(en)(C3,O8-CPD_2)] (**19**) crystallizes

in the triclinic space group P1 with the following cell parameters: a = 10.528(2), b = 11.533(2), c = 7.728(1) Å, α = 89.91(1)°, β = 106.25(1)°, γ = 70.92(1)°, V = 841.0 Å^3, Z = 2. The structure was solved as in ref 9, using 2098 reflections ($2\theta < 45°$). Refinement of the absorption corrected data, with all atoms (except H) assigned anisotropic thermal parameters converged at R_f = 0.019 and R_w = 0.029. Full details will be reported at a later date.

15. The test protocol for the S180a screen is described in ref 16. Compounds were administered on day one using 6 mice/dose; an ILS > 50% indicates activity. The reported dose is that which gave the the best %ILS. The test protocol for the L1210 screen is given in ref 17. Compounds were administered on day one and an ILS > 25% indicates activity. Surviving mice were counted as dying on the day of evaluation.
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18. Data not shown.
19. Abbreviations: CHD, 1,3-cyclohexanedione; MCHD, 5-methyl-CHD; DCHD, 5,5-dimethyl-CHD; CPD, 1,3-cyclopentanedione; CPD_2, CPD dimer.
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A COORDINATION CHEMICAL APPROACH TO PREPARE ORGAN-SPECIFIC ANTITUMOR PLATINUM COMPLEXES IN CANCER CHEMOTHERAPY

Y. Kidani

INTRODUCTION

Since the discovery of the antitumor activity of cis-Platin by Rosenberg et al. in 1969, it has become one of the most useful antitumor agents in cancer chemotherapy. With the view to develop superior 2nd generation Pt complexes to cis-Platin, without kidney and unfavorable toxicity, the author is conducting a coordination chemical approach to prepare organ-specific antitumor complexes. Metal complexes are represented by the chemical formula,

(Carrier Ligand)---(Pt)---(Leaving Group)

Considering from the characteristics of metal complexes, it will be possible to prepare various kinds of complexes with a variety of physicochemical and physiological properties, by the modification of carrier ligands, central metal atoms and leaving groups. Based upon the concept, the author attempts to prepare superior 2nd generation antitumor Pt complexes with specific affinity to certain organs(1).

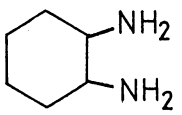
The author will describe mainly the preparation of

1) water-soluble D-glucuronate 1R,2R-cyclohexanediamine(=1R,2R-dach) Pt complexes,
2) development of oxalate 1R,2R-dach Pt complex,
3) oxalate and malonate 1R,2R-dach Pt(IV) complexes, and
4) lipo-soluble tetra-O-acetyl-α, and β-D-glucuronate 1R,2R-dach Pt complexes.

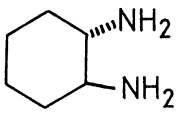
METHOD

The author employed 1,2-cyclohexanediamine(=dach) isomers and

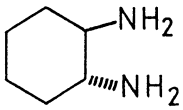
2(aminomethyl)cyclohexylamine(=amcha) isomers as carrier ligands and the central atom is fixed as Pt.



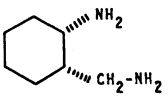
cis-dach (1R,2S-dach)



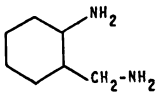
trans-d-dach (1S,2S-dach)



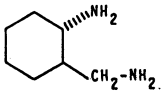
trans-l-dach (1R,2R-dach)



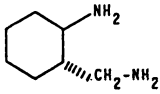
cis-d-amcha (1S,2S-amcha)



cis-l-amcha (1R,2R-amcha)



trans-d-amcha (1S,2R-amcha)



trans-l-amcha (1R,2S-amcha)

Antitumor screening was carried out at the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo with a kindness of Dr. Tazuko Tashiro, according to the Pt Analog Study Protocol, (leukemia L1210, 10^5 cells/CDF_1 mouse, ip-ip, on days 1, 5 and 9, one group = 6 mice).

RESULTS

Antitumor activity of $PtCl_2$(dach isomers) and $PtCl_2$(amcha isomers) showed that 1R,2R-dach was the most active ligand and the author prepared mainly 1R,2R-dach Pt complexes.

1. Preparation of water-soluble Pt complexes.

a. Monodentate carboxylate Pt complexes. Preparation of D-glucuronate 1R,2R-dach Pt complexes.

In order to prepare water-soluble 1R,2R-dach Pt complexes, the author took up sugar-carboxylate, β-D-glucuronate and D-gluconate as leaving groups. The reaction of $Pt(NO_3)_2$(1R,2R-dach) with β-D-glucuronate in 1:1 and 1:2 afforded Pt(D-glucuronato)(1R,2R-dach)·NO_3 (ℓ-GHP) and Pt(D-glucuronato)$_2$(1R,2R-dach) (=ℓ-G_2HP), both of which are highly water-soluble, >500 mg/ml. D-gluconate Pt complexes prepared are also very soluble in water, but the antitumor activity was not superior to D-glucuronate Pt complexes. Antitumor activity of ℓ-GHP, Table 1, Fig. 1, was found to be superior to cis-Platin and the effective against cis-Platin resistant tumors. Acute toxicity in Table 2 and subacute toxicity of ℓ-GHP indicated

Table 1 Antitumor Test Results for Pt(D-glucuronato)(trans-*l*-dach)·NO_3, *l*-GHP

tumors	Dose(mg/kg)	Schedule(days)	T/C%(cured)
L1210 leukemia	12.5	1, 5, 9	313(4/6)
	50	5, 9, 13	288(3/6)
P388 leukemia	12.5	1, 5	203
Lewis lung	2.5	q2dx10	155
B16 melanoma	3.12	1, 3, 5, 7, 9	168
	50	1, 5, 9	175

Fig. 1 Antitumor Activity of Pt(glucu)(trans-l-dach) Complexes against Leukemia L1210

(10^5 cells/CDF_1 mouse, by ip administration on days 1,5,9)

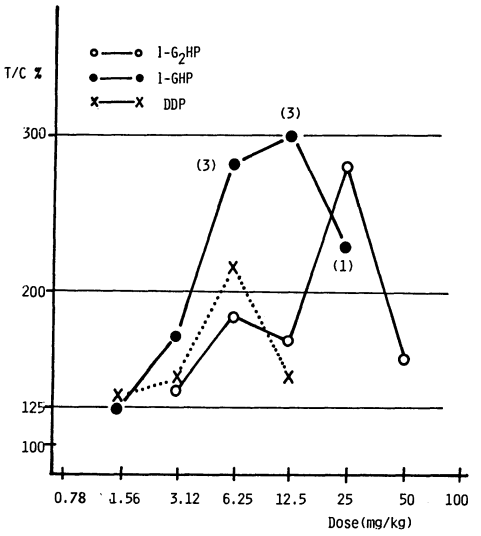
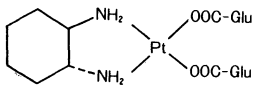


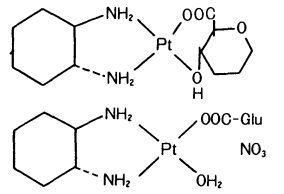
Table 2 Acute Toxicity

LD_{50}	1-GHP	rat	m	56.6 mg/kg	f	33.6 mg/kg
		mouse	m	52.7 mg/kg	(ip)	
	1-G_2HP	rat	m	56.0 mg/kg	(ip)	



Glu=D-Glucuronate

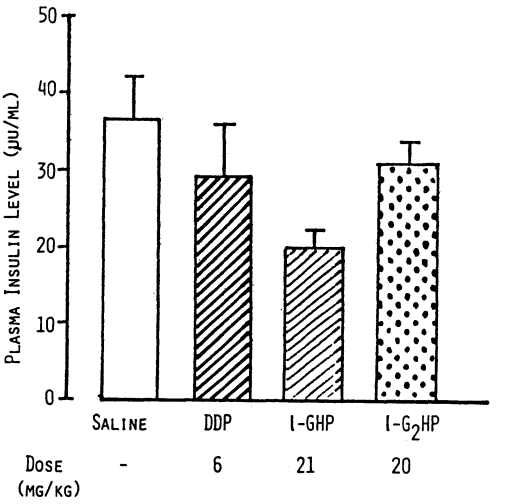
Pt(D-glucuronato)(trans-*l*-dach) Nitrate, *l*-GHP



Pt(D-glucuronato)$_2$(trans-*l*-dach), *l*-G_2HP

an inhibition of insulin secretion in blood, Fig. 2, but no kidney toxicity was observed. ℓ-G_2HP did not show any inhibition and was found non-mutagenic. Otherwise, future occurance of cancer by the treated antitumor agents may be suspected.

Fig. 2 INSULIN IN BLOOD



b. Bidentate carboxylate Pt complexes. Preparation of oxalate 1R,2R-dach Pt complex.

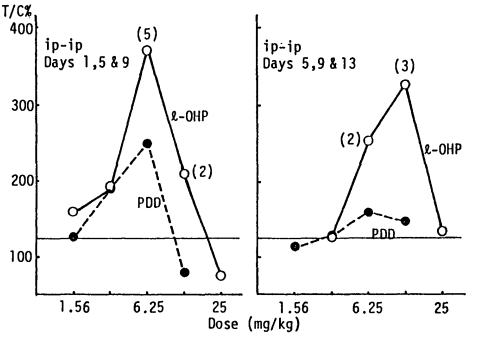
Though ℓ-GHP and ℓ-G_2HP are very soluble in water, their stability in aqueous solution was not high enough. Therefore, the author prepared bicarboxylate complexes to make Pt complexes more stable. For this purpose, chelates of oxalate and malonate were prepared. The solubility of malonate, Pt(mal)(dach) (=MHP) was very poor (0.23 mg/ml), though it showed high efficacy against acute granulocytic leukemia in Phase I Trials at the Wadley Institute of Molecular Medicine,Dallas, Texas, and the later clinical trials had not been proceeded, because of the poor solubility. The author prepared Pt(oxalato)(1R,2R-dach) (=ℓ-OHP). It is soluble in water (7.9 mg/ml). Secondary antitumor activity tests were carried out at the Cancer Chemotherapy Center and it indicated superior activity to cis-Platin, Table 3, Figs. 3, 4, 5, especially against cis-Platin resistant strains. Combination chemotherapy with adriamycin and cyclophosphamide indicated very promising effect, Figs. 6, 7. It is very stable in an aqueous solution for more than a week, measured by means of HPLC and a half life in saline solution was 11.6 hr. It is non-mutagenic by the Ames Test. Prof. G. Mathé in France paid a great interest of developing ℓ-OHP clinically(2) and Phase I and II clinical trials are now in progress. ℓ-OHP did neither show any kidney

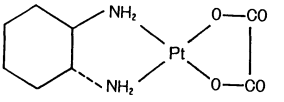
Table 3 Antitumor Test Results for Pt(oxalato)(trans-*l*-dach)

Tumors	System	Schedule	Dose (mg/kg)	Life Span (cures) T/C %	Tumor Weight T/C %
L1210 leukemia	ip-ip	1, 5, 9	6.25	320(3/6)	
			3.12	380	
L1210/DDP leukemia	ip-ip	1, 5, 9	6.25	380(6/6)	
			3.12	380(6/6)	
Lewis lung	sc-ip	q2dx10	2.5	145	29
B16 melanoma	ip-ip	1, 5, 9	10	200	
	sc-ip	1, 5, 9	10	107	11
			5	128	31
Colon	ip-ip	1, 5	12.5	143	
Colon 38	sc-ip	1, 8	10		16
MX-1	sc-ip	1	12.5		18

Fig. 3

SCHEDULE-DEPENDENCY OF ANTITUMOR ACTIVITY OF PDD AND OXALATO(TRANS-ℓ-DACH)Pt(II) AGAINST L-1210 (ip-ip)

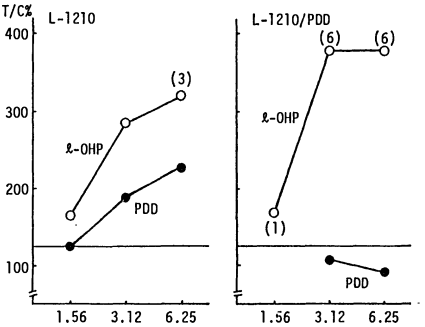




Pt(oxalato)(trans-*l*-dach), *l*-OHP

Fig. 4

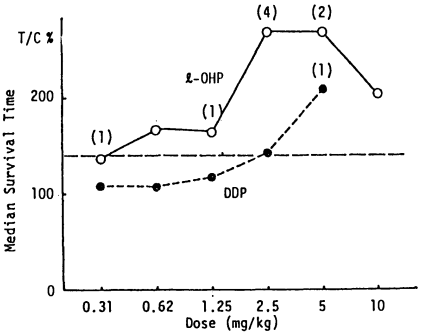
ANTITUMOR ACTIVITY OF PDD AND ℓ-OHP AGAINST PDD-SENSITIVE AND -RESISTANT L-1210 IN VIVO



Treatment : ip-ip, days 1, 5 & 9

Fig. 5

ANTITUMOR EFFECT OF ℓ-OHP AND DDP ON M-5076 (IP - IP)



Treatment : Days 1, 5 & 9
Numbers in parentheses denote cured mice evaluated on day 90

toxicity nor hematologic toxicity, and nausea and vomiting are the dose limiting factor(30 mg/m^2), and they are attenuated by the administration of antiemetics, Plitican. No inhibition of insulin secretion was observed, Fig. 8.

Phase I trials indicated good responces in metastatic breast cancer, lung cancer, melanoma, hepatocarcinoma and colorectal carcinoma(3), and Phase II trials indicated the complete respon-

Fig. 6

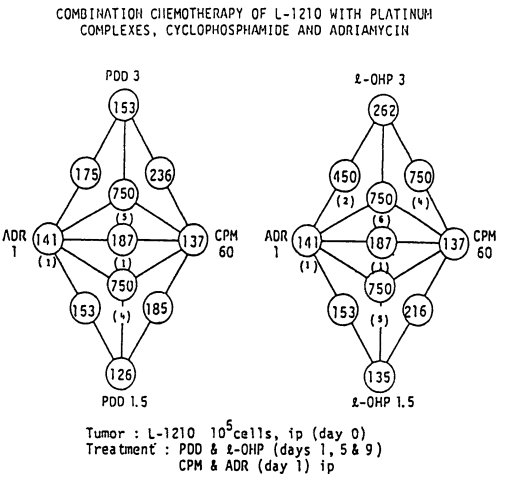


Fig. 7

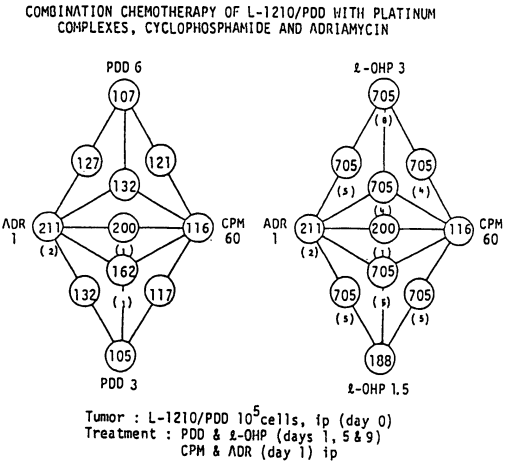
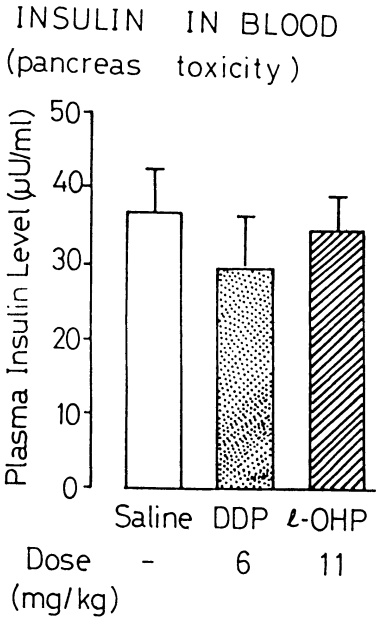


Fig. 8



ses of cis-Platin resistant ovarian cancer. Evaluation of efficacy was not the principal aim of Phase I, but some very intersting and encouraging responses were observed. ℓ-OHP is considered to be one of the most effective and promising 2nd generation Pt complex, being effective other than cis-Platin ineffective cancer.

c. Change of valency. Preparation of Pt(IV) complexes of ℓ-OHP and ℓ-MHP.

Because of the poor solubility of MHP, the development had not been proceeded. The author prepared $PtCl_2$(mal)(1R,2R-dach) (=ℓ-MHP·Cl) and $PtCl_2$(ox)(1R,2R-dach) (=ℓ-OHP·Cl). Solubility of the former became much soluble (8.7 mg/ml). Both Pt(IV) complexes are stable in solid state, but they are photoreduced to the respective Pt(II) complexes in an aqueous solution. They are antitumor active, Table 4.

Table 4

Antitumor Activity of Pt(IV) Complexes of dach Isomers ogoinst L1210 (2)

Complexes	Dose / mg/kg								
	400	200	100	50	25	12.5	6.25	3.12	1.56
	T/C %								
$PtCl_2$(ox)(trans-l-dach)				133	330(3)	311(3)	244(1)	197(1)	135
$PtCl_2$(mal)(trans-l-dach)			0	243(2)	286(2)	221(2)	127	105	103

2. Preparation of lipo-soluble Pt complexes. Preparation of O-acetyl-α and β-D-glucuronate Pt complexes.

Lipo-soluble antitumor agents are considered to be effective against the cure of brain tumor. Therefore, the author prepared lipo-soluble 1R,2R-dach Pt complexes. The author synthesized tetra-O-acetyl-α and β-D-glucuronate, and tetra-O-acetyl- and penta-O-acetyl-gluconate, and mono and bis 1R,2R-dach Pt complexes were prepared. Expectedly, mono Pt complexes are soluble both in water and chloroform, but bis Pt complexes are not soluble in water, but soluble in alcohol, benzene and chloroform. They showed very high antitumor activity, Table 5.

Table 5

Complexes	Toxic Dose mg/kg	Optimum Dose mg/kg	Optimum Dose T/C %	MED* mg/kg	MED* T/C %	T.I.**
Pt(Ac_4-α-glucuro)(dach)·NO_3	100	25	283(2/6)	3.12	184(1/6)	8
Pt(Ac_4-α-glucuro)$_2$(dach)	200	50	382(5/6)	3.12	128	16
Pt(Ac_4-β-glucuro)(dach)·NO_3	100	25	375(5/6)	1.56	132	16
Pt(Ac_4-β-glucuro)$_2$(dach)	200	50	382(4/6)	6.25	128	8

*MED: minimum effective dose **T.I.: therapeutic index

The numbers in parentheses indicate cured mice on 30-day observation.

CONCLUSION

Considering from the author's concept, it will be possible to prepare various Pt complexes by the modification of carrier ligands and leaving groups. Empirically, 1R,2R-dach is the most effective carrier ligand and preparation was made mainly on 1R,2R-dach as a carrier ligand. By the modification of leaving groups, it was found possible to prepare various complexes intentionally, such as

1) highly antitumor active complexes,
2) less toxic complexes without kidney toxicity, attenuating nausea and vomiting,
3) different distribution in body, and
4) different physicochemical properties, such as solubility and stability.

Finally, the author prepared Pt complexes by the modification of leaving groups with vitamins and hormones to afford organ-specific complexes. The author prepared vitamin B_2 derivatives Pt complexes and steroid-containing Pt complexes. The study is in progress. Steroid hormones employed are corticosteroid hormones, mele hormones and femalehormones.

The coordination chemical approach will surely be beneficial for further development in cancer chemotherapy.

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METAL-COORDINATION CHEMISTRY WITH BIOMOLECULES: PLATINUM-NUCLEOBASE INTERACTIONS

B. Lippert, J. Arpalahti, O. Krizanovic, W. Micklitz, F. Schwarz and G. Trötscher

INTRODUCTION

This lecture is intended to point out, from an inorganic chemist's view, some aspects of Pt coordination chemistry involving nucleobases. Most of the studies I will be refering to, have been done with models and therefore, their relevance might be considered limited. However, models developed by coordination chemists, have strongly influenced the thinking of scientists from various disciplines as, on the other hand, observations of biochemists have encouraged inorganic chemists to try to interpret these findings by modeling.

A major task for inorganic chemistry still is the synthesis of new compounds, their characterization and proper purification, both with regard to provide models that might explain biological effects, and to get new compounds that might be superior in their activity. In particular, metal complexes with a high specificity for tumors, as could possibly be achieved by linking them to monoclonal antibodies, is a goal of high priority.

In the following, mainly platinum-nucleobase interactions will be discussed, but it is likely, that quite a few of the aspects raised will be equally relevant to other metals or metal compounds.

PREPARATIVE AND STRUCTURAL ASPECTS OF METAL-NUCLEOBASE COMPLEXES

Mono- and Multinuclear Pt Complexes*

Pt(II) binding to the heterocyclic part of a nucleobase may formally be considered in terms of number of nucleobases N bound per platinum (n = N/Pt), e.g. n = 4, 3, 2, 1 or less (Table 1). While

Table 1. Different Stoichiometries of Pt and Pt,M Complexes Containing Nucleobases

n [a]	Example [b]	Reference
4	$[Pt(9\text{-MeG})_4]^{2+}$	10
3	$[Pt(NH_3)(1\text{-MeC})_3]^{2+}$	11
	$cis\text{-}[(NH_3)_2Pt(1\text{-MeC})_2]^{2+}$ x 1-MeC	12 [c]
	$cis\text{-}\ (NH_3)_2Pt(9\text{-EtG}^-)_2$ x 9-EtG	13 [d]
2	$cis\text{-}[(NH_3)_2Pt(9\text{-EtG})_2]^{2+}$	14
1.5	$cis\text{-}[(NH_3)_4Pt_2(9\text{-EtG}^-)(9\text{-EtG})_2]^{3+}$	15
1.33	$cis\text{-}[(NH_3)_4Pt_2Ag(1\text{-MeU})_4]^{+}$	16
1	$cis\text{-}[(NH_3)_2Pt(1\text{-MeC})Cl]^{+}$	17
	$cis\text{-}[(NH_3)_2Pt(1\text{-MeU})]_2^{2+}$	18 [e]
0.80	$cis\text{-}[(NH_3)_8Pt_4Ag(1\text{-MeU})_4]^{5+}$	19
0.66	$cis\text{-}[(NH_3)_4Pt_2Ag(1\text{-MeU})_2]^{3+}$	20
0.50	$trans\text{-}[Cl_2Pt(i\text{-prop})_2SO]_2(9\text{-MeA})$	3
0.33	$[(NH_3)_9Pt_3(9\text{-EtG}^-)]^{5+}$	8

[a] n = number of nucleobases per metal

[b] one example for each case given only

[c] third nucleobase is costacked with a coordinated nucleobase

[d] third nucleobase is hydrogen-bonded to a coordinated nucleobase

[e] dinuclear complex

the maximum number of bound nucleobases is given by the normal coordination number 4 of Pt(II), low n values are limited by the number of donor sites available at the nucleobase. Additional possibilities arise if heteronuclear Pt_xM_y complexes are included in this scheme.

Formation of multinuclear complexes is particularly versatile and therefore shall be considered in more detail. Basicity of a donor atom is a or even the major determinant of metal binding to a nucleobase.[1] Pt(II) binding to a neutral nucleobase reduces its overall basicity (H^+ affinity) and increases the acidity of other protons. For example, N7 Pt(II) binding to adenine makes the N1 position less basic by a factor of $10^{2.5}$.[2] This loss of basicity is not sufficient, however, to prevent formation of diplatinated, N7,N1 bridged complexes,[2-4] which then show a markedly increased acidity of the exocyclic amino protons ($pK_a \simeq 11.3$).[4] Although Pt(II) behaves similar to other transition metals in this respect,[5] there is also the possibility that a coordinated metal may increase the basicity of a neutral heterocyclic ligand through charge back-donation from the metal into the ring.[6]

With N7 platinated guanine, the acidity of the N1 proton has increased by ca. $10^{1.6}$,[7] thus making the N1 site more susceptible to additional metal binding. Likewise, primary metal binding to the deprotonated N1 position of guanine increases the Brönsted basicity of the ring as a whole since the metal, unlike the proton, permits the negative charge to be spread across the heterocycle. Eventually metal coordination may even take place at the N3 position, thus leading to a complex with Pt(II) moieties at N7, N1, and N3.[8]

The phenomenon of an increase in metal affinity of a singly metalated nucleobase anion, first discovered by Simpson in a study of CH_3Hg^+ binding to nucleobases,[9] and later interpreted by Tobias

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* Abbreviations: 1-MeU(1-MeT) = 1-methyluracil(1-methylthymine) anion; 1-MeUH(1-MeTH) = neutral 1-methyluracil(1-methylthymine); 1-MeC = neutral 1-methylcytosine; 1-MeC^- = anionic 1-methylcytosine,deprotonated at N4; 1-MeC^{2-} = anionic 1-methylcytosine, doubly deprotonated at N4; 9-EtG = neutral 9-ethylguanine; 9-EtG^- = 9-ethylguanine anion; 9-MeA = neutral 9-methyladenine; 9-MeA^- = anionic 9-methyladenine,deprotonated at N6; 9-MeAH^+ = 9-methyladenine cation; 1-MeU-N3 = metal coordination through N3, etc.

and coworkers,[21] also applies to the 1-MeU and 1-MeT systems: With cis-$(NH_3)_2PtL_2$ (L = 1-MeU or 5-substituted derivative), protonation to give cis-$[(NH_3)_2PtL(LH)]^+$ is facilitated by 4-4.5 log units relative to the unplatinated, neutral ligand.[22] As a result of increased ligand basicity, the formation of multinuclear complexes with these pyrimidine nucleobases is observed with di-,tri-,tetra-, penta-, and polynuclear species structurally characterized.[23]

Finally, formation of multinuclear complexes with metals bound through exocyclic amino groups, as observed with CH_3Hg^+ and 1-MeC^-, 1-MeC^{2-}, and 9-MeA^{2-} is noteworthy.[24] There, the N-metal bonds are formed in condensation reactions between a M-OH moiety and amino protons. Both Pt(II) [25] and Pt(IV) [26] have been found to undergo similar reactions with 1-methylcytosine.

Models of Cisplatin-DNA Adducts

Modeling Pt-DNA interactions at various degrees of complexity has been a major goal of bioinorganic chemistry research. What is a good model? As far as binding sites are concerned, any metal complex with metal coordination other than N1 of pyrimidine bases and N9 of purine bases is relevant. As to geometric aspects of metal-nucleobase cross-links, a good model should conform to the feasible arrangement of the nucleobases involved. Consider the GG cross-link of Cisplatin: Only if the two bases are oriented head-to-head, they are in an orientation to be expected in double-stranded DNA.

As demonstrated by den Hartog et al,[27] sophisticated NMR is capable of providing good information of the overall geometry of a particular cross-linking product, including an estimation of the dihedral angle between the base planes (53 ^{o} for the d(GpG) adduct). Crystal structure determinations of three GG cross-links, with 9-EtG,[14] d(pGpG),[28] and d(CpGpG),[29] give somewhat larger angles of 68 - 78 ^{o}, 76 - 87 ^{o}, and 80 - 84 ^{o}, respectively, suggesting that the actual distortion of DNA by this cross-link is larger than anticipated on the basis of NMR. Comparing the solid-state structures of the three GG cross-links, it is evident that models as simple as alkylated nucleobases (9-EtG) may indeed mimic a dinucleotide fairly well. However, there is unfortunately no guarantee of obtaining "real" models using these unconnected bases: Replacing the ammines

in cis-$[(NH_3)_2Pt(9\text{-}EtG)_2]^{2+}$ by propylamine ligands gives a product with head-tail arranged guanines.[30]

Although the GG adduct is the preferred cross-link, there is evidence from immunological studies that the in vivo adducts might indeed be more heterogeneous than concluded from in vitro studies.[31] It consequently is reasonable to also consider other cross-linking products of Cisplatin. Of all structurally studied models,[32] only two - cis-$[(NH_3)_2Pt(1\text{-}MeC\text{-}N3)(9\text{-}EtG^{-}\text{-}N7)]^{+}$ and cis-$[(NH_3)_2Pt(1\text{-}MeT\text{-}N3)(9\text{-}MeAH\text{-}N7)]^{2+}$ - have the alkyl groups in the proper orientation to satisfy the above mentioned criterion of a good model.[33,34] In either case, however, the purine nucleobase adopts a syn conformation.

Metal-Nucleobase Chelates*

Pt(II) chelate formation with nucleobases, specifically with guanine, has been discussed controversially for many years. At present, the N7,O6 chelate has not been proven unequivocally in the case of Pt(II). However, with bis(cyclopentadienyl)titanium(III), the N7,O6 chelate has been observed by Cozak, Beauchamp and co-workers both with xanthine[35] and theophylline.[36] The xanthine complex is unique in that it also contains a 4-ring-chelate involving N1 and O2 and in addition has a monofunctionally bound $(Cp)_2Ti(III)$ unit at N9 (Figure 1). The only unambiguously proven Pt nucleobase chelate is that of Pt(IV) with $1\text{-}MeC^{-}$, which involves N3 and N4.[26] In this 4-ring-chelate, angles about the Pt metal strongly deviate from the normal 90 ^{o}, which suggests that angles at the metal may be subject to considerably higher variations than anticipated in previous discussions of the N7,O6 chelate. Moreover, Pt-N bonds deviate markedly from the expected lone pair directions. The only prerequisite for chelate formation appears to be nucleobase deprotonation, hence an increase in donor basicity. The resulting strong M-donor bonds apparently outweigh unfavorable angles both at the heterocycle and the metal. As demonstrated with the $1\text{-}MeC^{-}$ chelate,

* This discussion is restricted to chelates involving donor atoms of the heterocyclic parts of nucleobases only, excluding semi-chelation, as observed in many Cu complexes of cytosine (N3,O2), and chelates of thioanalogues of nucleobases.

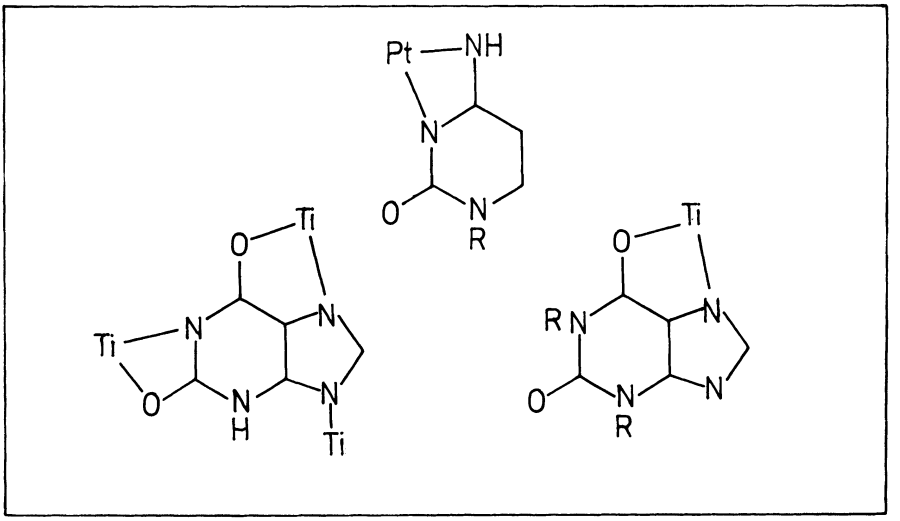

Figure 1. Schematic representations of metal chelates involving deprotonated forms of cytosine (top), xanthine (left) and theophylline (right).

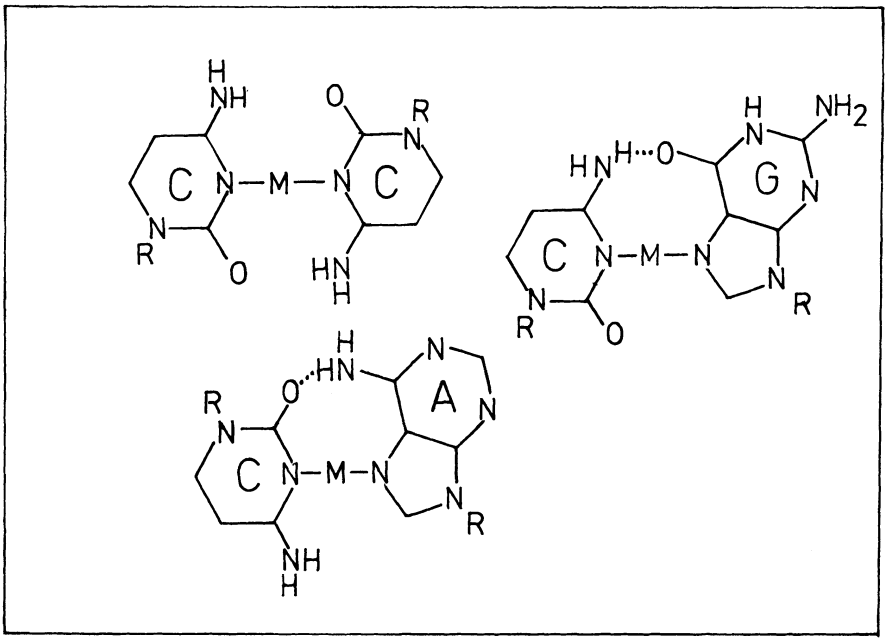

Figure 2. Metal analogues of $[CH^+] \equiv C$, $[CH^+] = G$ and $[CH^+] = A$ base pairs: A hydrogen bond is replaced by a N-M-N covalent bond.

nucleobase deprotonation can be achieved intramolecularly by a Pt-OH group already coordinated via N3. Similar reactions might be anticipated for a large series of metal nucleobase complexes and lead to a number of new chelates, including N1,N6 chelates of adenine, and N1,N2 or N1,O6 chelates of guanine, possibly even the disputed N7,O6 chelate.

Metal-Modified Base Pairs

Trans-$(NH_3)_2Pt(II)$ nucleobase complexes have not received great attention, primarily because trans-$(NH_3)_2Pt(II)$ compounds are inactive as antitumor agents. At least from an academic point of view, trans-Pt(II) complexes involving one pyrimidine and one purine nucleobase or two pyrimidine nucleobases are attractive, however, in that they represent metal-modified base pairs: If, for example, the acidic proton in hemiprotonated cytosine, $(CH^+) \equiv C$, is replaced by the linear $(NH_3)_2Pt$ fragment, the metal-modified analogue is obtained. As a consequence of the increase in N3 positions in the Pt analogue (from ca. 3 Å to ca. 4 Å), hydrogen bonding between the exocyclic groups of the two cytosines is no longer possible.[37] In contrast, H bonding is maintained between O6 of G and N4 of C in trans-$[(NH_3)_2Pt(9\text{-EtG-N7})(1\text{-MeC-N3})]^{2+}$,[32] which is the formal analogue of the $G_{syn} = (CH^+)_{anti}$ base pair.[38] Similarly, H bonding between N6 of A and O2 of C in trans-$[(NH_3)_2Pt(9\text{-MeA-N7})(1\text{-MeC-N3})]^{2+}$ leads to an almost coplanar arrangement of the two nucleobases,[2] a feature that might possibly be exploited to develop base-specific metal complexes (Figure 2).

Metals and Base-Mispairing

Metals or metal complexes may, by a variety of ways, impair the proper transfer of genetic information and cause mutations.[39] Three obvious possibilities would be (i) prevention of proper base pairing through blocking of hydrogen bonding sites, (ii) a shift of tautomer equilibrium as a consequence of metal binding, and (iii) a change in nucleobase protonation state.

The first possibility is trivial and needs not to be considered further: Any metal coordination at a site normally involved in Watson-Crick base pairing prevents correct pairing.

As to the second possibility, X-ray crystallographic and spec-

troscopic evidence exists that rare nucleobase tautomers, ordinarily present in proportions of 10^{-4} to 10^{-5} relative to the preferred tautomer, can be stabilized by metals through coordination at a lone electron pair: The 4-imino,2-oxo tautomer of cytosine has a Pt(IV) moiety at its N4 group,[40] while the 4-hydroxo,2-oxo tautomer of uracil and thymine is platinated at N3.[22] Neither of the two compounds represent good models for base-mispairing schemes, since as in (i), the position of the metals (N3 in T; N4 in C, syn to N3H) prevents even mispairing. However, cleavage of the Pt-N3(thymine) bond, as observed in solution,[22] might eventually lead to base-mispairing, as could conceivably a metal coordinated to N4 of cytosine trans to N3H.

The number of possible homo- and hetero-base pairs of the four common base pairs (28, assuming a minimum of two H bonds) further increases if protonated or deprotonated nucleobases are allowed. The involvement of protonated nucleobases in mismatched base pairs is now well established.[38,41] Probably the most important message to be taken from these findings is that pK_a considerations should be used cautiosly when dealing with polynucleotides or small fragments thereof. Nucleobase mispairing as a consequence of deprotonation of a nucleobase has been proposed and questioned,[42] but definitely been proven with N7 platinated, N1 deprotonated 9-ethylguanine. The following mismatches have been observed in crystal structure determinations and, in part,[7] confirmed in solution studies:
(i) mispairing between Pt(9-EtG$^-$-N7) and Pt(9-EtG-N7)[43]
(ii) mispairing between Pt(9-EtG$^-$-N7) and 9-EtG [13]
(iii) mispairing between two Pt(9-EtG$^-$-N7) moieties.[13]
While (i) and (ii) form three hydrogen bonds between each other, two H bonds are involved in (iii).

Isolation of Models

Dealing with models of metal nucleobase interactions raises the question as to whether all feasible compounds can be obtained in crystalline form and what the biological relevance of an isolated compound with respect to the solution behavior is. There is ample evidence that most of the Pt-nucleobase binding patterns, found in crystal structure determinations, indeed represent the solution

behavior. This is a consequence of the high thermodynamic stabilities of the complexes formed between Pt and nucleobases. With metal complexes of low thermodynamic stability, the respective solubilities of the (model) nucleobase and the complex formed can be crucial: Even if formed in solution, crystallization of the complex may become impossible because of low solubility of the free nucleobase, which shifts the equilibrium M + N ⇌ MN completely to the left. This situation appears to apply, for example, to the 1:1-complexes of neutral, N9-substituted guanines (9-MeG, 9-EtG, guanosine) with first row transition elements which, unlike their 5'-GMP analogues, are rather sparse. A crude guide line for crystallization of a particular complex using slow evaporation of the solvent is given in Table 2.

REACTIVITY ASPECTS OF PLATINUM-NUCLEOBASE COMPLEXES

Binding Site Recognition and Metal Migration

Quantitative studies of the types of cross-links formed between cis-$(NH_3)_2Pt(II)$ and enPt(II) with DNA have shown, that two thirds of all bound Pt end up in an intrastrand GG cross-link.[44,45] This percentage is by far too high to be explained by a random first reaction at any G of DNA (assuming approximately equal proportions of the four common bases) and subsequent reaction with a second G adjacent to the first one. Only after short incubation times, the amount of Pt bound to GG sites is consistent with the random platination model,[45] while a high percentage is bound monofunctionally and can be trapped by nucleophiles such as proteins,[46] thiourea,[45] (^{14}C)-guanosine,[47] or NH_3 (from NH_4HCO_3).[48] How does Pt recognize its preferred binding site? Are there transient adducts[49] and if so, how do they rearrange to give predominantly the GG clip? It has been suggested by Eastman,[45] that the initial, monofunctionally bound Pt might dissociate again from DNA and, in search for GG sites, "walk" along DNA.

Pt-GG Recognition: A Hypothesis. According to our present knowledge, protein-DNA recognition is brought about by specific hydrogen bonds formed between Watson-Crick base pairs (donors and acceptors in the major and minor groove of DNA) and amino acid side

Table 2. Qualitative Guide for Isolation of Metal Nucleobase Complex

complex stability	solubility [a]		isolation
	nucleobase	complex	
high			yes
low	good	poor	yes
low	poor	good	no

[a] good and poor used in relative terms

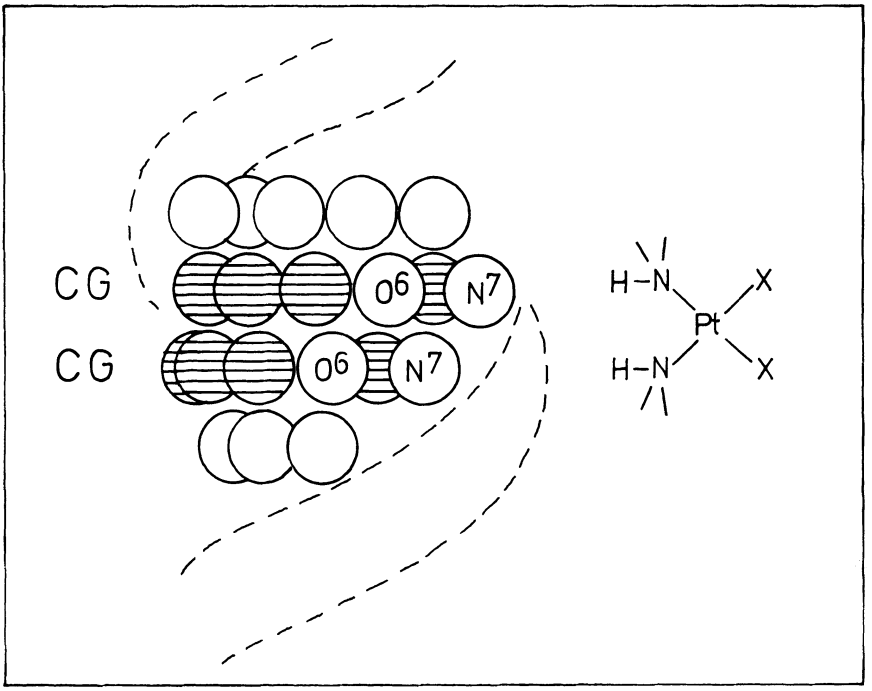

Figure 3. Schematic view of major groove of DNA with adjacent guanines. Only O6 and N7 of each CG base pair are hydrogen acceptors. AT base pairs have hydrogen acceptors (O4 of T and N7 of A) that are not next to each other as are O6 and N7 of guanine.

chains, in addition to other interactions (possibly intercalation of aromatic amino acid residues).[50] Similarly, sequence-specificity of typical groove-binding antibiotics such as netropsin and related compounds is attributed to H bonding with A,T stretches in the minor groove. Could it be possible that in a similar way Cisplatin finds its GG sites? When cis-Pt(II) approaches DNA through the major groove, it sees a lot of hydrogen acceptors (Figure 3), specifically G-N7, G-O6, A-N7, T-O4 and the phosphate oxygens from the backbone. With two or more adjacent guanines, a patch of four or more H bonding sites would be formed. Potential H donors (A-N6, C-N4) are comparatively few in numbers. In principle, cis-Pt(II) might use a proton from either amine group, from aqua ligands, or a combination of both to sense the major groove for suitable acceptor sites before it actually covalently binds to the two G-N7 sites. The utilization of amine protons for this job seems particularly attractive in that it might be this function rather than stabilizing the formed Pt-nucleobase complex through H bonding with either O6 of G[29,51] or a phosphate oxygen[28,52] which makes at least one proton at each amine so important in order to achieve antitumor activity.

Speculation is stopped here, however, not without encouraging colleagues from biochemistry to keep this interesting aspect in mind by quantifying DNA adducts of cis-Pt(II) derivatives which do not possess N-protons, such as cis-$(pyridine)_2PtCl_2$ etc. Any reduction in GG specificity might be support for the above hypothesis.

Reactivity of Pt-Nucleobase Bonds. How reactive is a Pt-nucleobase bond? How easy is it for Pt to "walk" along DNA once it is fixed?

Pt(II) nucleobase complexes involving endocyclic N donors are thermodynamically very stable, exceeding the corresponding Pd(II) complexes, for which data are available.* Due to the generally slow kinetics of reactions of Pt(II), bond cleavage is also not expected to be favored from a kinetic point of view. Own studies on the reactivity of a series of model nucleobase complexes (2.5×10^{-4} M)

* For dienPd(II)-5'-nucleotide complexes, log K values are as follows: T-N3, 8.7; G-N7, 8.1; G-N1, 7.9; C-N3, 5.5; A-N1, 4.9; A-N7, 4.1.[53]

of composition cis-$(NH_3)_2PtL_1L_2$ (with L_1L_2 = $(1\text{-MeC})_2$; $(9\text{-EtG})_2$; 1-MeC, 9-MeA-N7; 1-MeC, 9-MeAH-N7; 1-MeC, 9-EtG; 1-MeT, 9-MeA) and trans-$(NH_3)_2PtL_1L_2$ (with L_1,L_2 = $(1\text{-MeC})_2$; 1-MeC, 9-MeA-N7) toward a 40fold excess of 5'-GMP^{2-} and inosine, respectively (37 °C, H_2O, pH 4.7-7) showed no reaction whatsoever within 100 h. Thus any substitution of a N-bound nucleobase by another one must be a very slow process. We tentatively conclude, that a sequence of nucleophilic substitution reactions as an explanation for Pt migration on DNA is unlikely if a Pt-N bond is involved.

Applying stronger nucleophiles such as thiourea or cyanide, nucleobase substitutions take place.[54,55] However, steric restriction may, as demonstrated for N3 platinated thymine and uracil,[54] lead to a surprising inertness of the Pt-nucleobase complex.

With oxygen-bound Pt (phosphate, exocyclic oxygens of bases), nucleophilic substitutions should be comparatively easy, but the question remains whether the entering nucleophile is bound weakly enough to be substituted by another ligand. For example, with dinuclear complexes of type cis-$[(NH_3)_2Pt(1\text{-MeU-N3,O4})]_2^{2+}$ (head-head or head-tail), the Pt-O bonds are readily cleaved by Cl^-,[56] NH_3 and 9-EtG, and the head-tail dimer exists in solution even in an equilibrium with the mononuclear aqua complex according to

$$(h\text{-}t)[(NH_3)_2Pt(1\text{-MeU-N3,O4})]_2^{2+} + 2H_2O = 2\ [(NH_3)_2Pt(1\text{-MeU-N3})H_2O]^+$$

Findings, that (^{14}C)-uracil of Pt-uracil-blue is not associated with the complex formed between the blue and DNA,[57] could be explained this way, assuming that the blue essentially consists of dinuclear head-head units.

Metal Migration on Isolated Nucleobases. Metal migration processes on nucleobases or related ligands, which involve cleavage of an initially formed metal-N(nucleobase) bond, have been reported in several instances. Examples include migrations of di- and tripositive $(NH_3)_5Ru$ moieties from N3 to N9 in hypoxanthine,[58,59] from N7 to C8 in 1.3-dimethylxanthine,[60] from N3 to N4 in cytidine,[61] from N1 to N6 in adenosine,[61] as well as migrations of dienPd(II) from N7 to N1,[53] and N3 to N4 in cytidine.[62]

With platinum, the following linkage isomerizations have been reported:

(i) N1-N3 and N3-N1 migrations of $(NH_3)_3Pt(II)$ at unsubstituted uracil,[63]
(ii) N7-N1 migration of dienPt(II) at inosine,[53]
(iii) N1-N7 migration on 9-EtG,[64]
(iv) head-head to head-tail isomerization in a pyridonato complex,[65]
(v) N3-N4 migration of Pt(IV) in 1-MeC.[40]
Of all examples, process (v) is best understood, because chelate intermediates have been isolated and structurally characterized.[26] Processes (i) and (ii) take place via dinuclear intermediates with Pt atoms simultaneously coordinated at N1 and N3 (uracil) and N1 and N7 (inosine), respectively, while (iv) starts out with a dinuclear complex. Process (iii) occurs only after protonation of the N1 platinated guanine at the N7 position and is comparable to cleavage of the Pt-N3(uracil, thymine) bond after protonation of the exocylic oxygen.[22]

In summary, it appears that Pt migration on a nucleobase requires binding of one or more electrophiles (metal, proton) to at least two sites of the heterocyclic ring. Considering the pH of most physiological media, it seems more likely that two metals rather than one metal and a proton might interact in a linkage isomerization process.

Unusual Antitumor Activity. Closely related to the question concerning the reactivity of a Pt-N(nucleobase) bond is the question as to why certain Pt compounds violating some of the rules considered vital for activity (neutrality, cis-configuration of two leaving groups), show in fact antitumor activity. Specifically, Pt(II) complexes having three "inert" ligands, e.g. three N donors in cis-$[(NH_3)_2Pt(pyridine)Cl]Cl$[66] or cis-$(NH_3)_2Pt(1\text{-}MeU)Cl$[67] or two N and one C donor in Pt(cis-dach)(ascorbate-C2,O5) (with dach = 1.2-diaminocyclohexane),[68] deserve mentioning in this context. While these compounds may be metabolized *in vivo* or possibly act in a way different from Cisplatin or its analogues, it certainly is interesting to note that with cis-$(NH_3)_2Pt(1\text{-}MeU)Cl$, for example, only protonation has been shown to produce a cis-$(NH_3)_2Pt(II)$ fragment with loss of 1-MeU from the complex. There may be a higher chance for the above compounds to lose a NH_3 ligand as a consequence of the trans-effect of

the Cl and carbon donors, as observed in the related cis-[$(NH_3)_2$Pt (1-MeC)Cl]Cl complex,[69] but then the species formed has a trans-geometry and would have to isomerize to yield a compound with two reactive functions in a cis-arrangement.

FEEDBACK TO INORGANIC CHEMISTRY

Up to now, this overview has been dealing primarily with studies that more or less emerged from questions asked by biophysisits, biochemists and colleagues from the medical sciences. There is at least one area of inorganic chemistry, in which this impulse has resulted in "new chemistry"[70] which already is an integral part of modern inorganic chemistry textbooks: Well defined, low-nuclearity mixed-valence Pt complexes and novel diplatinum(III) compounds. They were a response to the question concerning the nature and composition of a class of antitumor agents reported by Rosenberg in the early seventies, the so called "platinum pyrimidine blues",[71] which at the same time revitalized the almost forgotten problem of "Platinblau".[72]

Mixed-Valence Compounds.

Unlike the Krogmann salts,[73] which lose their mixed-valence character in solution through dissociation into discrete, mononuclear Pt(II) and Pt(IV) entities, the "Pt pyrimidine blues", prepared by reaction of cis-$[(NH_3)_2Pt(H_2O)_2]^{2+}$ with uracil, thymine, cytosine, and related cyclic amidate ligands, maintain their mixed-valence character in solution,[74] unless they undergo chemical reactions, e.g. oxidation of water.

The group of Lippard has structurally characterized three representatives of the Pt(2.25) type "blue",[75] which consists of two stacked head-head dimer units with the four Pt centers interconnected by metal-metal bonding. Of the four metals, three are in the formal +2 oxidation state, while the fourth one is in +3. The Pt(2.25) blue is generated by chemical (e.g. HNO_3) or electrochemical oxidation of the head-head diplatinum(II) complex.[76] As to the former, it has now been demonstrated, that metals such as Fe^{3+}, Ce^{4+}, and Ag^+ are also capable of oxidizing diplatinum(II) complexes, and in the case of Ag^+, a heteronuclear Pt_2Ag intermediate has been

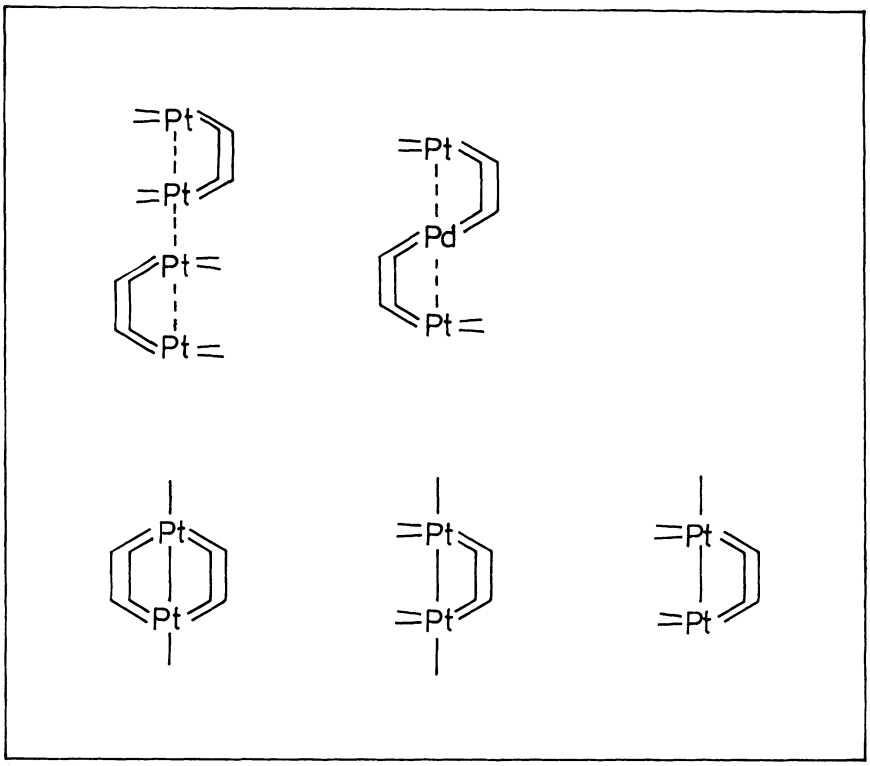

Figure 4. Mixed-valence tetra- and trinuclear complexes containing uracil (and thymine) bridges (top) and diplatinum(III) complexes (bottom) with four and two bridging ligands and hexa- and mixed hexa,penta-coordination of metals.

crystallized, which appears to be a direct precursor of the Pt(2.25) blue.[20]

Using α-pyrrolidone as the bridging ligand, additional mixed-valence compounds of average Pt oxidation states 2.14, 2.37, and 2.5 have been isolated by Matsumoto and coworkers.[77] As with the Pt(2.25) blue, head-head dimers are the building blocks in these complexes.

Finally, a novel trinuclear platinum blue "analogue", consisting formally of two Pt(II) and one Pd(III), linked by four 1-MeU or 1-MeT rings through N3 and O4 each, has been described by Micklitz et al.[78] Complexes of type cis-$[(NH_3)_2PtL_2PdL_2Pt(NH_3)_2]^{3+}$ are paramagnetic (1.9 B.M.) and intensely blue with absorptions around 600, 550, and 350 nm. The schematic structure of these trinuclear blues is given in Figure 4. It is to be compared with the structures of Pt(2.25) blue and the variuos diplatinum(III) structures (vide infra).

Diplatinum(III) Complexes. Pt coordination chemistry has long been dominated by the classical square-planar Pt(II) and octahedral Pt(IV) complexes. The existence of diplatinum(III) complexes with hexa-coordination of the two metals and formation of a Pt-Pt single bond has first been proven in 1976 by a Russian group.[79] Since then, a considerable number of other diplatinum(III) complexes have been prepared and characterized.[80] The list of structurally characterized compounds not only includes examples with four bridging ligands, but also derivatives of cis-$(NH_3)_2Pt$ with two bridging ligands only, with different coordination numbers of the two Pt atoms (5 and 6),[81] and different combinations of axial ligands (c.f. also Figure 4).[82]

CONCLUSION

Coordination chemistry with nucleobases offers a wide field of activities for interested chemists, ranging from synthesis (metal complexes with improved biological activity, models of metal-nucleic acids interactions) to crystallography, spectroscopy, and physico-chemical studies (stability, kinetics, metal-metal interactions). Research with the metal Pt has provided deeper insight into the basic chemistry of this element (e.g. mixed-valency, Pt-O bonds) and contributed to the understanding of biological effects of antitumor Pt complexes.

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CHEMICAL CONSIDERATIONS IN THE DESIGN OF RUTHENIUM ANTICANCER AGENTS

M.J. Clarke, R.D. Galang, V.M. Rodriguez, R. Kumar, S. Pell and D.M. Bryan

ABSTRACT

Ruthenium compounds offer a promising approach to the development of new anticancer agents that attack the same initial sites (G^7) as platinum, but are more likely to undergo redox chemistry *in vivo*. Several free ruthenium complexes induce DNA strand cleavage, probably through Fenton's chemistry. Purine oxidation and hydrolysis reactions of N(7)-bound $[(NH_3)_5Ru^{III}]$-nucleoside complexes occur simultaneously in the mid pH range. Linkage isomerization occurs in adenosine complexes and has been re-investigated by square-wave voltammetry. The N(6) to N(1) isomerization specific rate for $[(NH_3)_5Ru^{II}]$ is 1.5 s^{-1} with activation parameters of $\Delta H^{\ddagger}$= 44.3 ± 0.5 kJ/mol and $\Delta S^{\ddagger}$ = -93.3 ± 3 kJ/mol °K. The reverse isomerization for Ru(III) occurs with a specific rate of 10.3 s^{-1} with activation parameters of $\Delta H^{\ddagger}$= 26.5 ± 0.5 kJ/mol and $\Delta S^{\ddagger}$ = -135 ± 11 kJ/mol °K. Ru(III) complexes of ascorbate and the related tetramethylreductic acid have been prepared and exhibit a pH-dependent reaction, which is most easily explained as a linkage isomerization with the metal moving from O(3) to O(1) on lowering the pH from neutral to acidic. The reverse O(1) to O(3) isomerization occurs on increasing the pH. While the ascorbate complex exhibited no antitumor activity, a similar squarate species yielded a T/C of 147 against P388 lymphocytic leukemia in mice. This difference in anticancer activity is attributed to the relative ease of reduction of Ru(III) in the two compounds.

INTRODUCTION

Over the past three decades a number of attempts have been made to develop ruthenium-containing pharmaceuticals.[1,2,3,4,5,6] Tissue distribution studies of several ^{103}Ru or ^{97}Ru-labelled complexes indicate a fair degree of tumor localization, which has been attributed to two, perhaps not unrelated, physiological mechanisms.[4,7,8]

Ruthenium occurs in aqueous solution predominantly as Ru^{II}, Ru^{III} or Ru^{IV}. The ions in the lower two oxidation states are almost invariably six coordinate with octahedral geometry and are generally inert to substitution when bound to nitrogen bases (with half-lives on the order of years).[9,10] The loss of ammines and heterocyclic nitrogen bases from $[L(NH_3)_5Ru^{II}]$ (where L = NH_3 or nitrogen heterocycle) is somewhat faster, but still proceeds fairly slowly with half-lives on the order of a day under physiological conditions.[11] Ruthenium(IV) compounds generally require oxo[12,13] or sulfido ligands for stabilization.[14]

The reduction potentials of Ru^{III} complexes vary considerably with the ligands present. In the relatively simple series of ions of the type $[L(NH_3)_5Ru^{III}]$, $E^{o'}$ values vary from -0.08 V with L = hydroxide to 0.9 V with a neutral flavin ligand.[15] In general, anionic, σ-donor ligands lower the reduction potential, while neutral or cationic, π-acceptor ligands raise it. Owing to the small changes in bond distances between ammineruthenium(II) and -(III) ions, the Franck-Condon barrier to electron transfer is small and redox reactions involving $Ru^{II,III}$ couples are usually rapid.[7,16] In the case of ammineruthenium(II) ions, ligand substitution is controlled by the rate of water exchange, which occurs with a half-life of about 0.1 sec.[8] In most ammineruthenium(II and III) complexes the bonds to the ammonia and other nitrogen ligands are sufficiently strong to remain intact following electron transfer. Consequently, once coordinated to a nitrogen base, either ion usually remains bound to the same base, if not to the same atom on the base.[7,9]

APPROACHES TO RUTHENIUM ANTICANCER AGENTS

While tumor localization is not a prerequisite for chemotherapeutic activity, in many cases it is desirable and is necessary for radiodiagnostic or radiotherapeutic pharmaceuticals. For ligands that are large with respect to the metal ion, localization is usually ligand-dependent; on the other hand, when a dominating ligand is not present, localization depends on the inorganic chemistry of the metal ion.

An elegant example of tumor localization resulting from protein labelling is ^{103}Ru-transferrin, whose uptake by the EMT-6 sarcoma in mice was almost twice as high as that of the most widely used tumor-imaging agent.[17,18] Neoplastic cells, especially those in rapidly growing tumors, have a high iron requirement and, consequently, a large number of receptors for the Fe-transport protein, transferrin. Since Ru is immediately below Fe in the periodic table and has a high affinity for phenolate ligands,[19] which are involved in the transferrin Fe-binding site,[20] it is not surprising that Ru also has a high affinity for this plasma protein.

A similar path has been taken with bleomycin, a tumor-seeking, iron-requiring, antibiotic used successfully as an adjuvant to cisplatin. Direct reaction of bleomycin with $RuCl_3 \cdot H_2O$ at 210 °C, followed by peroxide oxidation, yielded a product that retained a level of toxicity to cells in tissue culture identical with free bleomycin and exhibited similar *in vivo* uptake by rat tumors.[21] Meares has reported on the photo-induced nicking of DNA in the presence of air by a Ru(II)-bleomycin, which was prepared by reduction of $RuCl_3 \cdot H_2O$ in basic ethanol at 200 °C.[22] In a separate study, $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ was combined with bleomycin at room temperature. Spectroscopic and electrochemical experiments indicated monoruthenium-bleomycin adducts with $[(NH_3)_5Ru^{III}]$ coordinated to either an imidazole ring nitrogen or to the exocyclic ammine of the pyrimidine moiety in a 1:3 ratio, which may vary with reactant conditions.[23] These complexes are cytotoxic, but unlike iron-bleomycin, failed to show any increased ability to cleave DNA in the presence of oxygen and a reductant. On the other hand, the complex exhibited significant tumor uptake and the ruthenium ion appeared to be released upon reduction. Preliminary studies indicated no significant chemotherapeutic activity.

A straightforward example of the second approach involves ammine-ruthenium(III) ions as prodrugs. This does not require the presence of specific receptor sites, but does rely on increased binding to tumors relative to normal tissue. Since ruthenium(III) ions are relatively "hard" and have a high attraction for halides and anionic oxygen ligands, these ligands are retained for fairly long periods. Conversely, ruthenium(II) ions are relatively "soft" and have little affinity for these ligands. As a result, prodrugs introduced into an organism as ruthenium(III) species with such acido ligands can be expected to remain fairly stable as long as the tripositive oxidation state is maintained. However, should these compounds become reduced, they immediately lose the acido ligand and are thrown into a rapid binding mode. Therefore, tissue binding is expected to be favored in areas low in oxygen and high in reductants, such as the reducing, hypoxic environment prevalent in many tumors.[24] Absorption of $RuCl_3 \cdot H_2O$ following ingestion appears to parallel that of iron. It is concentrated by the villi of the small intestines and then widely distributed, with a maximum blood concentration occurring within 12 hr.[25] A portion remains in the blood for a relatively long period and may be presumed to be coordinated to transferrin.[17,18] Redox processes that play a role in iron transport may be involved and transfer of the metal ion into the cell may be dependent on a transmembrane electron transport mechanism, which reduces the metal ion and thereby facilitates its loss from transferrin.[26]

Overall, it is likely that tumor accumulation of simple ammineruthenium complexes proceeds by two pathways. First, rapid tumor uptake proceeding through activation of the Ru^{III}-prodrug toward binding by reduction in the tumor. Since small ions are excreted fairly readily by the kidneys, this mode of binding should decrease rapidly with time. A second, slower mode of tumor binding, which may occur for many days following injection, is probably mediated by transferrin.

Hepatic-specific complexes of ruthenium(III) have been designed with iminodiacetato ligands containing lipophilic groups, which facilitate uptake by the biliary tract.[27] Ruthenocene and its lipophilic derivatives are excreted in both urine and bile following hydroxylation in the liver and formation of a glucuronide conjugate[28] and some ruthenocene derivatives have been designed to localize in the adrenals and other organs.[29,30] The complex $[(pyal)(NH_3)_5Ru^{III}]^{3+}$ (pyal = b-(4-pyridyl)-a-alanine) shows good uptake by the pancreas.[31] Complexes with phosphate and phosphonates can be incorporated into bone.[32] Ruthenium red[33] binds *in vitro* and *in vivo* to cell surfaces high in acidic glycoproteins and inhibits calcium uptake. It has been used to image tumors[2-4,34] and inhibits DS sarcoma cells *in vitro*.[35] Hexaammineruthenium(III) causes the elimination of pBR322 and pBR329 plasmids from E. coli.[36]

DNA BINDING

In vivo there are a number of possible reduction mechanisms to activate Ru(III) prodrugs to bind in the Ru(II) state. While mitochondria will slowly catalyze the NADH reduction of ammineruthenium(III) ions, microsomes are considerably more efficient.[37] Conversely, whole mitochondria will not oxidize $[(NH_3)_6Ru]^{3+}$, but cyt-b_{562} on the inner face of submitochondrial particles and cytochrome oxidase will.[38,39] This complex is also reduced by the transmembrane electron transport system, which has been present in all cells tested.[26] In general, these rates are rapid, if the electron-transfer site on the protein is near the surface. If the redox site is deeply buried within the protein matrix, the rates are several orders of magnitude slower.[40]

Binding of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ to proteins occurs on exterior histidinyl imidazoles, with a surprisingly high degree of selectivity.[41,42,43] While $[(NH_3)_5Ru^{III}]$ has a high affinity for sulfhydryl groups,[44] these complexes are often unstable and, when this ion is coordinated to sulfido groups on proteins and the adduct usually reverts to starting materials.[45] Complexation of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ to amine groups on proteins, such as surface lysines, appears to be inhibited by competition from protons. The affinity of this ion for carboxylate moieties is inherently low.[40]

Binding of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to both DNA[46] and RNA[47] has been observed, but is quite slow. Coordination of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ to DNA follows fairly rapid biphasic kinetics and is relatively independent of DNA concentration in the millimolar range of $[P_{DNA}]$. Initial binding occurs primarily at the N(7) of guanine (G^7), which is relatively exposed in the major groove of B-DNA. A likely mechanism for ruthenium(II) binding involves an initial ion-pairing equilibrium between the DNA polyanion and the metal cation. This concentrates the metal at the surface of the DNA and increases the efficiency of the reaction. Rapid binding follows dissociation of the water molecule on $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$. The overall rate law for this pathway is then:

$$\frac{d[Ru\text{-}G]}{dt} = \frac{f_G k_3 k_2 K_{ip}}{(k_{-2} + k_3)(1 + K_{ip}[P_{DNA}])}[P_{DNA}][Ru^{II}]$$

where k_2 is the water substitution rate on $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$. The effective DNA concentration is equal to that fraction of sites which are G, i.e. $[DNA]_{eff} = f_G \times P_{DNA}$. K_{ip} is a function of the ionic strength of the monocations in the solution and can be estimated to be around 200 at $\mu = 0.1$ and 900 in TA buffer.[48]

At $[Ru^{II}]/[P_{DNA}] > 0.5$ or with single-stranded DNA, spectroscopic bands arise due to coordination to the exocyclic amines, N(6) of adenine and N(4) of cytosine.[49] K_{assoc} for helical calf thymus DNA has been determined to be 5.1×10^3 and 7.8×10^3 for denatured DNA. Binding at low $[Ru^{II}]/[P_{DNA}]$ causes a linear decrease in T_m and a decrease in the circular dichroism spectrum, which suggest a weakening of the DNA helix. The reduction potential for $[(NH_3)_5Ru^{III}]$ at G^7 on DNA is 48 mV.[46]

Sundarlingham, *et al.* showed that binding of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to phenylalanine transfer RNA resulted in both ion-paired and covalently bound adducts.[46,50] Direct coordination was observed at the N(7) of G_{15}, which was further stabilized by hydrogen bonding between ammine protons and O(6) and phosphate oxygens on P_{14} and P_{15}. Additional N(7) binding occurred at G_1 and G_{18}. The G_{15} and G_{18} sites are in non-helical regions, where their N(7)'s are well exposed and the phosphate charge density is high.[47]

Barton has shown that Ru(II) complexes with large, bidentate aromatic ligands intercalate into DNA to a degree dependent on their chirality and that of the nucleic acid.[51] Enantiomers of $[(DIP)_3Ru]^{2+}$, where DIP = 4,7-diphenylphenanthroline, distinguish between right- and lefthanded DNA helices, with the D-enantiomer preferentially binding to right-handed B-DNA. While both enantiomers bind equally to left-handed Z-DNA, space-filling models suggest that a left-handed DNA with a

tighter helix should preferentially bind the Λ- enantiomer.[52] Direct coordination (probably at G^7) has been proposed with similar complexes such as *cis*-$[Cl_2(phen)_2Ru^{II}]$; however, in this case it is the Λ-enantiomer which selectively binds B-DNA.[51] Binding proceeds somewhat slowly ($t_{1/2}$= 1.5 h) and is probably preceded by intercalation through the *o*-phenanthroline rings.

LINKAGE ISOMERIZATION

Metal ion migration of pentaammineruthenium has been shown with purine and nucleoside ligands. Movement between the N(3) of 7-methylhypoxanthine and the nearby N(9) site has been observed with a half-life of about 1.5 hr at low pH, but at a negligible rate at a pH above the pK_a of the complex, where deprotonation from N(1) leaves a negative charge on the pyrimidine ring.[53] Initial coordination of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ to adenosine is presumed to be mainly at the endocyclic, N(1) site. Oxidation to Ru^{III} facilitates deprotonation of the adjacent exocyclic ammine, N(6), making this the more attractive binding site, so that the metal ion easily moves to form an amide complex.[4,54] The reverse N(6) to N(1) movement of the metal ion can be observed by reducing the N(6)-Ru(III) complex, since backbonding with the resulting Ru(II) is favored by coordination to the adjacent endocyclic imine site.

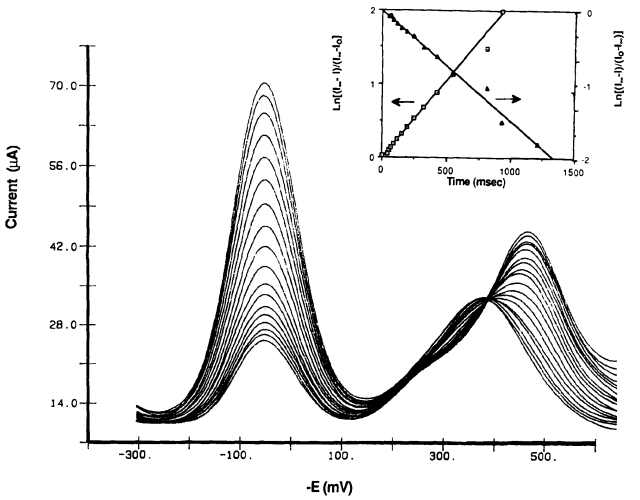
Since proton equilibria were hypothesized to play an essential part in these mechanisms, it was necessary to find reductants to extend the study over a broad pH range. Initially, the exo → endo isomerization was studied by stopped-flow spectrophotometry following reduction of 6-$[(5'\text{-AMP})(NH_3)_5Ru^{III}]$ with Cr^{2+} or Eu^{2+}. Over the pH range 1-5, the metal ion was observed to shift positions with a specific rate of $1.6 \pm 0.3\ s^{-1}$. Electrochemical methods allow for precise control of the reduction potential at any pH; however, interfering electrochemical processes prevented accurate measurements of the rates by cyclic voltammetry (CV). Nevertheless, this approach did reveal the trend in the estimated rate constants to change markedly in the expected pH range.[4] For the exo → endo reaction to occur, a proton must replace the metal on the exocyclic nitrogen. This occurs most readily when the proton is already available on the complex at N(1). Above the pK_a (≈11.3) the reaction ceases. Similarly, the reverse (endo → exo) isomerization was predicted to proceed more readily in a pH range above the predicted pK_a (7-9) of 1-$[(Ado)(NH_3)_5Ru^{III}]$, where the metal ion is attracted by the anionic N(6). Again, the CV method revealed the rates to accelerate in this pH range, but did not yield precise values.

A new technique, square-wave voltammetry (SWV),[55] has now been applied with apparent success. In this method the desired oxidation state of the complex, is prepared *in situ* by a short electrolysis time at a stationary electrode. A varying amount of time is waited before determining the relative concentration of the electrolyzed or isomerized form by SWV. Since SWV is a differential method that is relatively insensitive to the background, it provides a current peak whose size is directly proportional to the analyte concentration.

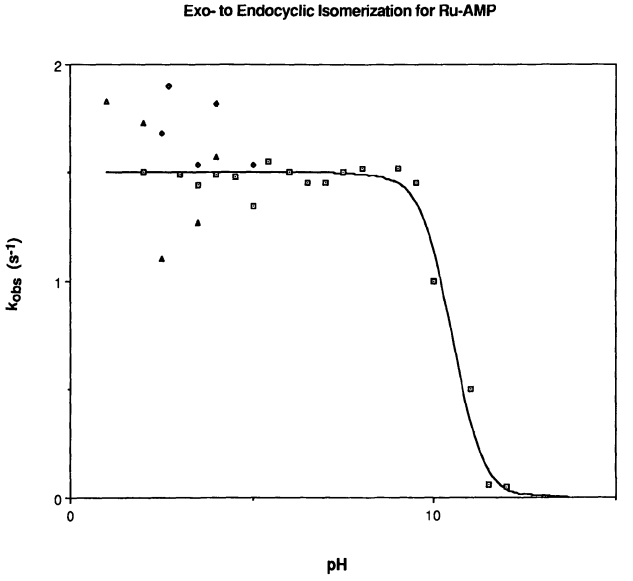
Figure 1 shows a series of such scans after increasing times of electrolyzing of 6-[(5'-AMP)$(NH_3)_5Ru^{III}$]. The peaks centered around -300 mV (relative to Ag/AgCl) are for the $Ru^{II,III}$ couple in 6-[(5'-AMP)$(NH_3)_5$Ru], which decrease with increasing reaction time, while those around 100 mV are for the corresponding couple in the N(1) isomer and increase with time. The inset shows the plots of $\ln(I_\infty - I_t)$ versus time for both isomers, where I is the SWV peak current for the appropriate isomer, to be linear. The rate constant obtained from the N(1) SWV peak is 1.5 ± 0.1 s^{-1}, while that from the N(6) peak is somewhat more rapid at 2.6 ± 0.8 s^{-1}. The slightly higher rate for the disappearance of the N(6) isomer can be accounted for by a small amount of dissociation of this complex to yield $[(H_2O)(NH_3)_5Ru]^{2+}$, which is seen in CV scans.[4] Figure 2 shows a plot of rate constants for the appearance of the N(1) isomer obtained by both stopped-flow spectrophotometry and SWV as a function of pH, revealing a "kinetic" pK_a to be similar to that for loss of a proton from the N(1) site of 6-[(Ado)$(NH_3)_5Ru^{II}]^{2+}$.

The reverse (endo $\rightarrow$ exo) movement of the metal ion can be observed by a double electrolysis step, followed by a fast SWV scan. Here 6-[(5'-AMP)$(NH_3)_5Ru^{II}$] is first prepared at the electrode surface by reduction of the Ru(III) analog. This is allowed sufficient time to isomerize to the N(1) species and is then electrochemically oxidized at a higher potential to yield 1-[(5'-AMP)$(NH_3)_5Ru^{III}$], which then back-isomerizes to the initial starting material. SWV following the second electrolysis quantitatively monitors the concentration of 1-[(5'-AMP)$(NH_3)_5$Ru] in the region of the electrode. The results of an experiment of this kind are shown in Figure 3. The plot of the observed rate constant as a function of pH is shown in Figure 4, revealing the kinetic pK_a to be in the predicted region ($\approx$8.2).

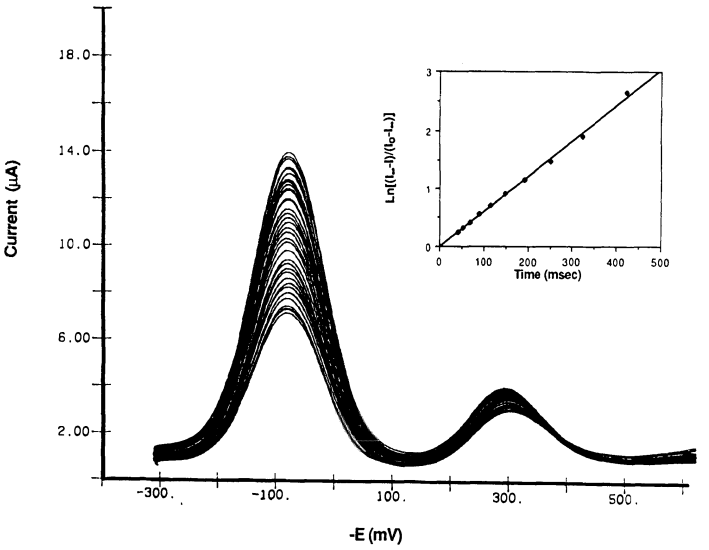
For the N(6) to N(1) reaction the activation parameters are: $\Delta H^{\ddagger} = 44.3 \pm 0.5$ kJ/mol and $\Delta S^{\ddagger} = -93.3 \pm 3$ kJ/mol °K. The reverse isomerization for Ru(III) occurs with a specific rate of 10.3 s^{-1} at 25°C with activation parameters of $\Delta H^{\ddagger} = 26.5 \pm 0.5$ kJ/mol and $\Delta S^{\ddagger} = -135 \pm 11$ kJ/mol °K. The large, negative entropies are consistent with an intramolecular reaction in which the metal ion is transiently bridging between



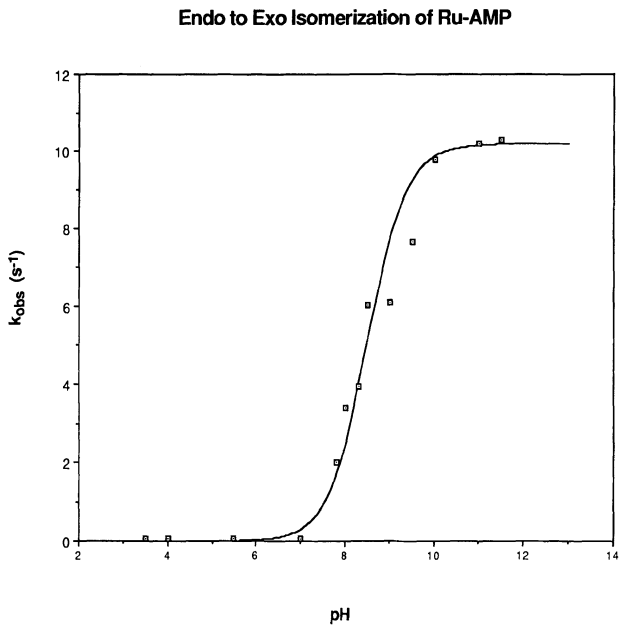
1. Square-wave voltammetry scans of a 6-[(5'-AMP)$(NH_3)_5Ru^{III}$] following varying electrolysis times at -600 mV (vs. Ag/AgCl). *Inset*: Plot of $\ln[(I_\infty - I_t)/(I_0-I_\infty)]$ for the peak centered around 100 mV versus time and $\ln[(I_t- I_\infty)/(I_\infty-I_0)]$ versus time for the peak at -300 mV.



2. Plot of k_{obs} versus pH for the exocyclic to endocyclic isomerization of 6-[(5'-AMP)$(NH_3)_5Ru^{II}$].



3. Square-wave voltammetry scans of a 6-[(5'-AMP)$(NH_3)_5Ru^{III}$] following 1 minute electrolysis at -600 mV (vs. Ag/AgCl) and varying electrolysis times at 350 mV. *Inset*: Plot of $\log[(I - I_t)/(I_o - I_\infty)]$ for the peak centered around 100 mV versus time.



4. Plot of k_{obs} versus pH for the endocyclic to exocyclic isomerization of 1-[(5'-AMP)$(NH_3)_5Ru^{III}$].

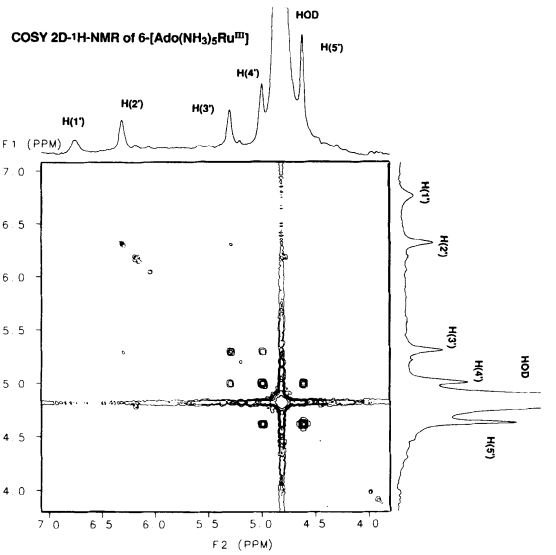
the N(1) and N(6) sites, thus losing some internal degrees of rotational freedom.

Owing to their paramagnetic character, Ru(III) complexes exhibit severely broadened and shifted ^{1}H-NMR resonances, which are also strongly pH dependent. The two-dimensional COSY FT-NMR technique provides a means of determining which protons are coupled and consequently allows for the assignment of the sugar protons. Figure 5 shows the 2-D COSY pattern for 6-[Ado$(NH_3)_5Ru^{III}$] with the appropriate assignments. The ring proton resonances, which are not shown, occur at δ = 23.3 ppm for H(8) and -15.7 ppm for H(2) at low pH. Interestingly, each of the ring proton resonances becomes more strongly broadened and divides into two peaks in the pH region around the spectrophotometric pK_a. A plot of these for H(8) is shown in Figure 6. The bifurcation of these peaks probably arises from two rotomeric forms of the complex: a) one in which the Ru atom is situated near N(1) and hydrogen bonds to this site through the ammine ligands, and b) a second in which the Ru is on the N(7) side of the molecule. The first is favored by deprotonation and the second should be forced upon protonation at N(1). As expected, both forms have different pK_a values (see Figure 6). Thus the metal ion on adenosine not only moves between adjacent sites as a function of the redox potential, but also swings from one side to the other dependent on the pH, when bound to N(6).

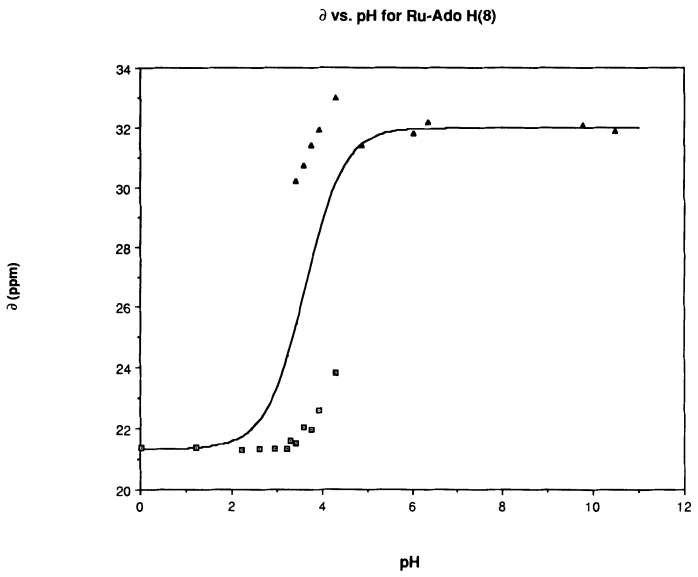
RUTHENIUM ASCORBATE COMPLEXES

Metal ions interact with ascorbate ion in a number of different ways. In at least one important enzyme, ascorbate oxidase, the coenzyme may bind directly with two Cu(II) ions at the active site.[56,57] Metal ions capable of undergoing redox reactions are thought to catalyze the autoxidation of ascorbic acid through the formation of intermediate metal-ascorbate-dioxgen complexes.[58] Such metal ions in the presence of ascorbate and oxygen are capable of efficiently cleaving DNA. Recently, an unusual platinum(II)-ascorbate complex was shown to have excellent antitumor properties.[59,60]

In an effort to delve into the actual electron-transfer to oxygen, in a manner which essentially precludes binding of dioxygen and subsequent inner-sphere electron transfer, we have synthesized a series of stable, monodentate complexes of ascorbic acid and tetramethylreductic acid, a related reductate ligand, and studied their reduction of dioxygen in aqueous solution. Tetramethylreductic acid[61,62] effectively mimics ascorbic acid in many of its redox properties, while being somewhat more tractable, especially in providing a stronger ^{1}H-NMR signal in paramagnetic Ru(III) complexes.



5. 2-D COSY spectrum of the sugar protons in 6-[Ado$(NH_3)_5Ru^{III}$] at low pH. Resonances not shown are H(8), ∂ = 21.35 ppm, and H(2), δ = -15.7 ppm.



6. Plot of δ vs. pH for H(8) on 6-[Ado$(NH_3)_5Ru^{III}$] illustrating the presence of pH-dependent rotomeric forms.

Both reductate ligands yielded two distinct complexes with $[(NH_3)_5Ru^{III}]$, which were separated chromatographically and are believed to be the O(2) and O(3) linkage isomers (see Figure 7).[63] ^{1}H-NMR spectra of the TMRA complex revealed the two forms to interconvert as a function of pH. The rate constant for the O(3) to O(2) isomerization is 8.0 x 10^{-4} s^{-1}. The subsequent dissociation of the O(2)-bound complex at pH 2.23 occurs with a specific rate of 1.0 x 10^{-4} s^{-1}. The half-life for the reverse O(2) to O(3) reaction at neutral pH has been estimated to be about 1-2 min. The loss of TMRA from the complex in neutral solution proceeds slowly with a rate of 3.3 x 10^{-5} s^{-1}.

Cyclic voltammetric scans for $[(TMRA)(NH_3)_5Ru^{III}]^+$ are shown in Figure 8. The reversible couple at positive potentials is attributed to the oxidation of the ligand, while the irreversible one occuring at negative potential is assigned to reduction of Ru(III) to Ru(II). The couple growing in is due to $[H_2O(NH_3)_5Ru^{II}]^{2+}$ arising from dissociation of the Ru(II) species following its production in the voltammetric scan. Additional electrochemical studies indicate that the reductate complexes provide a single-electron on oxidation. However, it is likely that the reductate ligand actually undergoes a net two-electron oxidation, with the second electron being transferred intramolecularly to the Ru(III), as has been observed with Ru(III) catecholato complexes.[64] A red, meta-stable species formed on oxidation of these complexes may then have the formulation $[L_{ox}(NH_3)_5Ru^{II}]^{2+}$, where L_{ox} is dehydroascorbate or dehydrotetramethylreductate.

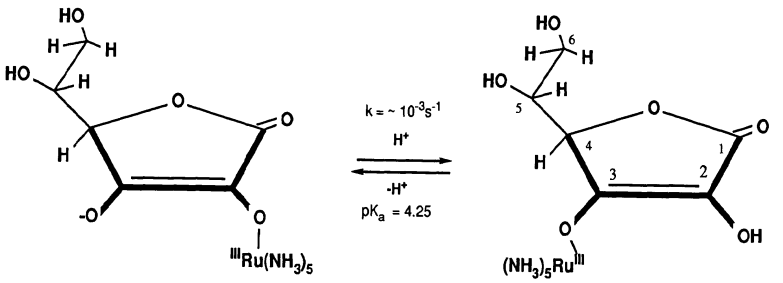
A wave centered at 16 mV was evident in cyclic voltammetric scans of a structurally similar complex with squararic acid, $[(C_4O_4)(NH_3)_5Ru^{III}]^+$, and is attributed to the reduction of the metal ion. This was almost completely irreversible on carbon paste electrodes at low pH, and only somewhat irreversible on platinum or carbon paste electrodes at neutral pH. Brief electrolysis of solutions at -100 mV to which isonicotinamide had been added revealed $[Isn(NH_3)_5Ru]^{2+}$ to form from the $[H_2O(NH_3)_5Ru^{II}]^{2+}$ released by the electrolysis.

The overall rate law for autoxidation of the reductate complexes is:

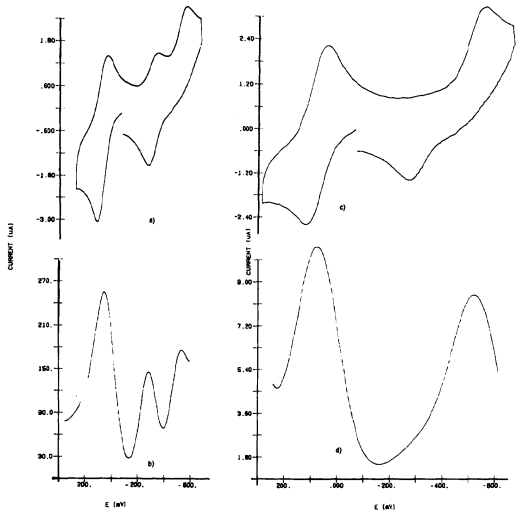
$$d[Ru\text{-}L]/dt = k[Ru\text{-}L][O_2]$$

where Ru-L is $[(Asc)(NH_3)_5Ru]^+$ or $[(TMRA)(NH_3)_5Ru]^+$. Specific rate constants at 25 °C for the ascorbate and TMRA complexes, respectively, are: 5.42 ± 0.2 sec^{-1} M^{-1} and 2.51 ± 0.06 $sec^{-1}M^{-1}$. Activation parameters are: $[(Asc)(NH_3)_5Ru]^+$, $\Delta H^{\ddagger}$ = 60.9 ± 2.3 kJ/mol, $\Delta S^{\ddagger}$ = -26.6 ± 1.2 J/mol°K; $[(TMRA)(NH_3)_5Ru]^+$, $\Delta H^{\ddagger}$ = 69.6 ± 1.7 kJ/mol, $\Delta S^{\ddagger}$ = -5.54 ± 0.16 J/mol°K.

Linkage Isomers of $[(Asc)(NH_3)_5Ru^{III}]$



7. Proposed linkage isomers of $[(Asc)(NH_3)_5Ru]^+$.



8. Cyclic voltammograms of $[(TMRA)(NH_3)_5Ru]^+$ in 0.15 M NaCl at pH 7 at a scan rate of 400 mV/s. Working electrode, Pt disk; reference electrode, Ag/AgCl (scale relative to this electrode). a) Scan showing $[(TMRA_{ox})(NH_3)_5Ru^{II}]^{2+} + e^- \rightleftarrows [(TMRA)(NH_3)_5Ru^{III}]^+$ couple and additional $[(TMRA)(NH_3)_5Ru^{III}]^+ + e^- \rightleftarrows [(TMRA)(NH_3)_5Ru^{II}]$ irreversible couple; b) Square wave voltammetry beginning at 300 mV and scanning cathodically illustrating presence of only the couples indicated in *a* and *b*; c) Subsequent scan illustrating the presence of the couple, $[(OH)(NH_3)_5Ru^{III}]^+ + e^- + H^+ \rightleftarrows [(H_2O)(NH_3)_5Ru^{II}]^{2+}$, which grows in as a result of dissociation of $[(TMRA)(NH_3)_5Ru^{II}]$, d) Square wave voltammetry beginning at -600 mV and scanning anodically showing couples indicated in *a*, *b* and *d*.

CHEMICAL EFFECTS OF Ru BINDING TO DNA

Either inner- or outer-sphere coordination to nucleic acids holds the potential for disrupting metabolism. Replication enzymes are sensitive to the ionic environment, so that even simple ion-pairing or hydrogen bonding may cause replication errors to be made. This may be especially so if such binding induces a local change in DNA conformation. More probable are adverse metabolic effects resulting from direct binding. Owing to the relative selectivity of ammineruthenium ions for the N(7) of purines, it is likely that these ions may concentrate in nuclear chromatin and so interfere with nucleic acid metabolism. Indeed, *in vitro* studies demonstrate that Ru^{II} and Ru^{III} compounds are active in inhibiting DNA synthesis[65] and possess mutagenic activity in the Ames and related assays.[66]

There are many possible mechanisms for interfering with DNA metabolism following binding to G^7 sites. These include: 1) failure of replicating enzymes to recognize the metallated G; 2) additional metal binding following or inducing helix disruption (as discussed above); 3) subsequent protein-, intra-, or interstrand crosslinking by the metal; 4) chemical reactions of the guanine residue induced by the presence of the metal ion; and, 5) Fenton's chemistry taking place at the metal ion to generate radicals capable of strand cleavage. Local helix disruption may be caused by a) the sheer bulk of the metal ion, b) weakening of the hydrogen bonding and π-stacking abilities of the base through polarization of electron density toward the metal cation, which is accentuated in the case of Ru^{III} by π-bonding effects, and c) O(6) and phosphate hydrogen bonding to ammine protons. Additionally, the linkage isomerization reactions already discussed may allow the metal to migrate on the surface of the DNA. The effect of $[(NH_3)_5Ru^{III}]$ on purine and pyrimidine acidity has been shown to vary in a predictable inverse-square fashion.[4,52]

Owing to the relatively high charge on Ru^{III}, chemical effects resulting from electronic polarization are often observed with this metal ion before becoming apparent with dipositive metal ions. For example, sugar-purine bond scission in $[(dG^7)(NH_3)_5Ru^{III}]$ has been measured with a half-life of 1.5 days at 56 °C and pH 7.[67] An unexpected reaction of $[(dG)(NH_3)_5Ru^{III}]$ is the air oxidation of the nucleoside to 8-hydroxydeoxyguanosine. Again, it is probably the π-bonding between the metal and nucleoside that facilitates both electron transfer to O_2 and deprotonation at the C^8 position that is thought to be involved in this oxidation mechanism.[65] This reaction proceeds with a half-life of about 1.8 hr at 56 °C and pH 7.56.

A number of relatively small complexes of redox-active transition metal ions have recently been shown to cleave DNA.[68, 69] At least several of these are engaged in an autooxidation process (Fenton's chemistry) that eventually produces hydroxy radicals with the metal ion being recycled through its reduced state, owing to the presence of a reductant. The hydroxy radicals generated proximally to the DNA are thought to attack the sugar moieties by hydrogen atom abstraction followed by sugar fragmentation and strand scission.[66] A number of ruthenium complexes havae been shown to cleave DNA, presumably through generation of Fenton's chemistry.[45] Surprisingly, direct binding of $[(NH_3)_5Ru^{III}]$ resulted in no strand cleavage above background. Nor was there any evidence for strand cleavage that should follow hydrolysis of the sugar-purine bond or oxidation of the C^8 of guanine.[45]

ANTITUMOR ACTIVITY

Ru-containing chemotherapeutic agents designed on the basis of the "activation by reduction" hypothesis have often exhibited good antitumor activity. Table 1 contains results for selected ruthenium complexes for anticancer activity. In general, a high percentage of the ruthenium coordination complexes tested show presumptive antitumor activity and are less toxic than cisplatin, but require a higher therapeutic dose. When mice bearing 180A sarcomas were treated with $[(Asc)(NH_3)_5Ru](CF_3SO_3)$ at various dosage levels, the following T/C values were obtained: 96%, 10 mg/kg; 103%, 20 mg/kg; 76%, 40 mg/kg; 38%, 80 mg/kg; 6%, 160 mg/kg; 6%, 320 mg/kg. The *cis*-dichlorodiammineplatinum control gave a T/C of 163% at 7 mg/kg. These results indicate the compound to be inactive in this tumor screen and toxic at the higher doses.

In the NCI antitumor panel, $[Sqr(NH_3)_5Ru]Cl\cdot H_2O$ proved to be effective against the mouse P388 lymphocytic leukemia tumor with a best T/C of 140% at a dose of 21.12 mg/kg, which was judged to be nontoxic with all animals surviving. The best T/C exhibited against L1210 lymphocytic leukemia was 130% at a nontoxic dose of 25 mg/kg. Against a melanoma, the compound exhibited a T/C of 126% at 6.25 mg/kg, with all animals surviving. However, the compound showed no effect against a MX1, transplanted human mammary tumor, nor against the M5076 sarcoma.

Since the squarate and ascorbate complexes are structurally similar, it is surprising that one shows good activity and the other does not. Assuming the target site to be chromatin nucleic acids, neither of these complexes would likely bind to these sites without ligand loss opening up a coordination position.[70] The electrochemical

Table I. Antitumor Activity of Selected Ruthenium Complexes.

Compound	Dose (mg/kg)	T/C (%)	Ref.
fac-$[Cl_3(NH_3)_3Ru]$	50	189	Clarke, 2
$[Cl_3(1,5\text{-dimethyltetrazole})_3Ru]$	80	179	Keller, Keppler,70
$[CH_3CH_2COO(NH_3)_5Ru]ClO_4$	12.5	163	Clarke, 2
$(ImH)_2[Cl_5ImRu]$ (Im = imidazole)	72.8	162.5	Keppler,71
$(1,2,4\text{-triazolium})[Cl_4(1,2,4\text{-triazole})_2Ru]$	45.1	161	Keller, Keppler,71
cis-$[Cl_2(NH_3)_4Ru]Cl$	12.5	157	Clarke, 2
$[(C_4O_4)(NH_3)_5Ru](F_3CSO_3)$	21.2	140	Pell, Clarke, 72
cis-$[Cl_2(DMSO)_4Ru]$	565	125	Sava, *et al.*, 73
$[Cl(NH_3)_5Ru]Cl$	1.5	116	Armor, 2
$[Ox(bipy)_2Ru]$	3.13	101	Clarke, 2
$[(Asc)(NH_3)_5Ru](F_3CSO_3)$	10	96	Pell, Clarke, 58
$[Cl_2(phen)_2Ru]ClO_4$	6.25	90	Clarke, 2

results show that both ascorbate and squarate ligands are rapidly lost on reduction of the ruthenium center. However, to be active in vivo the complexes must have biologically accessible reductions potentials. Indeed, the $Ru^{III,II}$ couple for the squarate complex is quite accessible at 16 mV; however, that for the ascorbate species (-300 mV) is at or below the limit available in a biological system. The difference between these reduction potentials indicates that it is much more likely that the squarate complex will be reduced *in vivo* than the ascorbate. Consequently, the squarate complex will produce a much greater quantity of $[H_2O(NH_3)_5Ru^{II}]^{2+}$, which

actively binds to nucleic acids.[74]

Ruthenium compounds present a particular promising and versatile path to pursue in the development of new chemotherapeutic drugs. Especially interesting would be soluble analogs of $[Cl_3(NH_3)_3Ru]$, a wider range of derivatives of *cis*-$[Cl_2(NH_3)_4Ru]Cl$, and complexes involving halides and nitrogen heterocycles. Recently issued patents indicate that compounds of the first and third types are being actively synthesized and tested.[75,76,77]

ACKNOWLEDGEMENT

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ORGANOMETALLIC TITANOCENE AND FERRICENIUM COMPLEXES: ANTITUMOR AND TOXICOLOGIC PROPERTIES

P. Köpf-Maier

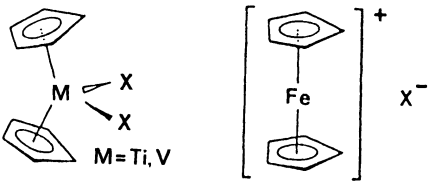
INTRODUCTION

Following the detection and clinical approval of cytostatic platinum complexes, antitumor activity was revealed during the past years for numerous other quite different inorganic and organometallic compounds. Metallocene and metallicenium complexes represent examples of organometallic compounds containing early or medium transition metals. Their antiproliferative activity has been detected in 1979 and 1984, respectively (1-3). Together with copper and gold complexes (4,5) and main group element compounds (6-9), metallocene and metallicenium complexes belong to the class of non-platinum group metal antitumor agents (10).

COMPOUNDS

Metallocene complexes are neutral compounds containing two cyclopentadienyl ring ligands in tilted position and two acido ligands X at adjacent sites. The main representatives of antitumor metallocene complexes are given (i) by metallocene dichlorides $(C_5H_5)_2MCl_2$ with titanium or vanadium as central metal atom M and (ii) by diverse titanocene derivatives $(C_5H_5)_2TiX_2$ modified at the position of the acido ligands X by introduction of various halide or carboxylato ligands.

On the other hand, metallicenium complexes are ionic, salt-like compounds. They include two cyclopentadienyl ring ligands in parallel position and do not contain covalently bound acido ligands. Antitumor metallicenium complexes are mainly represented by ferricenium compounds $[(C_5H_5)_2Fe]^+X^-$, containing iron as central metal atom. The anion X can be trichloroacetate, picrate, tetrachloroferrate(III) or μ-oxo-bis[trichloroferrate(III)].



ANTITUMOR ACTIVITY

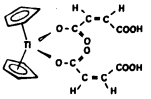
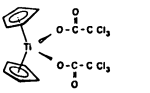
Experimental tumors.

Titanocene and ferricenium complexes are characterized by a similar spectrum of antitumor acivity against experimental animal tumors (Tables 1,2). Both effected marked antitumor activity against fluid Ehrlich ascites tumor and ascitic sarcoma 180, whereas there was no or only marginal activity against the murine leukemia systems L1210 and P388 (11). On the other hand, the proliferation of various solid experimental tumors growing subcutaneously such as sarcoma 180, melanoma B16, colon 38 carcinoma and Lewis lung carcinoma was suppressed significantly (12,13). Against most of these tumors superior activity was revealed for titanocene dichloride which induced growth inhibitions by 70-80 % to T/C ratios of 30-20 %. Another interesting representative of antitumor metallocene compounds is given by vanadocene dichloride. Besides its efficacy against fluid and solid Ehrlich ascites tumor (2,11), it showed pronounced activity against several other experimental tumor systems such as mouse mammary tumor (TA3Ha) and human epidermoid carcinoma (HEP-2) cells (14,15).

Human tumors.

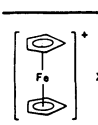
Human malignant tumors can be heterotransplanted into athymic mice and treated there with cytostatic drugs. Remarkably, there exists a high correlation between the response or non-response of human tumor xenografts to chemotherapy and the clinical results with the same drugs. When titanocene and ferricenium complexes were applied to athymic mice bearing various human tumors, significant growth suppression was observed in the case of colorectal carcinomas (16), lung malignancies (17), a breast and cervix carcinoma and a melanoma (Tables 3,4). The growth inhibitions which were effected amounted to 50-80 %, leading to T/C values of 50-20 %. Generally, these growth suppressions remained stable beyond the end of the

Table 1: Optimum therapeutic effects of titanocene complexes against experimental animal tumors

	Fluid tumors (ILS)				Solid tumors (Inhibition of tumor growth)				
	L1210	P388	Ehrlich ascites tumor	Sarcoma 180	Ehrlich ascites tumor	Sarcoma 180	B 16 melanoma	Colon 38 carcinoma	Lewis lung carcinoma
Control	0	0	0	0	0	0	0	0	0
	26	30	480	184	86	77	80	81	71
	22	24	480	128	81	63	74	63	66
	n.d.	n.d.	480	125	n.d.	47	50	63	43
	n.d.	n.d.	480	96	n.d.	63	31	52	n.d.

Given are optimum values of increase in life span (ILS, %) for fluid tumors or of growth inhibition in % of control tumor size (calculated by 100 % - T/C) for solid tumors, determined on key-dates.

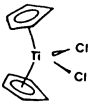
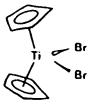
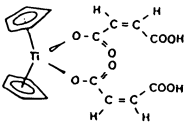
Table 2: Optimum therapeutic effects of ferricenium complexes against experimental animal tumors

	Fluid tumors (ILS)			Solid tumors (Inhibition of tumor growth)				
	L1210	P388	Ehrlich ascites tumor	Ehrlich ascites tumor	Sarcoma 180	B 16 melanoma	Colon 38 carcinoma	Lewis lung carcinoma
Control	0	0	0	0	0	0	0	0
$X^- = [CCl_3COO]^-$ · 2 CCl_3COOH	0	0	500	58	50	60	73	70
$X^- = \frac{1}{2}[Cl_3FeO\text{-}FeCl_3]^{2-}$	0	0	388	56	35	55	73	59
$X^- = [FeCl_4]^-$	0	0	364	56	43	65	57	31
$X^- = [2,4,6\text{-}(NO_2)_3C_6H_2O]^-$	0	0	500	17	48	19	62	66

See explanation to Table 1.

treatment period. Figure 1 illustrates the proliferation behavior of the adenocarcinoma S90 derived from the colon sigmoideum under the influence of titanocene dichloride in comparison to 5-fluorouracil which represents a clinically approved cytostatic drug showing efficacy especially against human colorectal carcinomas. There was no

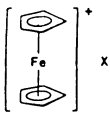
Table 3: Growth inhibitions [a)] of human xenografts by titanocene complexes

	Colon adeno-carcinoma	Rectum adeno-carcinoma	Breast carcinoma	Lung adeno-carcinoma	Lung small cell carcinoma
Control	0	0	0	0	0
	77	55	78	65	61
	69	35	69	46	57
	28	n.d.	n.d.	65	75

a) Given are optimum inhibition values in % of control tumor size (calculated by 100 % - T/C) 3 or 4 days after last substance application. Substance application according to Q3Dx5 or Q2Dx5. Applied doses correspond to LD_{10} regimens.

better activity by 5-fluorouracil in the case of S90 than by titanocenes. Analogous findings became evident investigating the response of several other adenocarcinomas from the colon including the colon sigmoideum and the rectum. In the case of 8 out of 10 tumors which were analyzed, titanocene dichloride induced growth inhibitions by 50-80 % resulting in T/C ratios of 50-20 %.

Table 4: Growth inhibitions[a] of human xenografts by ferricenium complexes

 x^-	Colon adeno-carcinoma	Rectum adeno-carcinoma	Breast carcinoma	Lung adeno-carcinoma	Lung small cell carcinoma
Control	0	0	0	0	0
$X^- = [CCl_3COO]^-$ · CCl_3COOH	37	67	0	52	40
$X^- = [CCl_3COO]^-$ · 2 CCl_3COOH	39	63	0	50	25
$X^- = \frac{1}{2}[Cl_3FeO\text{-}FeCl_3]^{2-}$	64	75	54	30	51
$X^- = [FeCl_4]^-$	67	77	30	71	n.d.
$X^- = [2,4,6\text{-}(NO_2)_3C_6H_2O]^-$	58	83	n.d.	57	n.d.

a) See explanation to Table 3.

The morphologic analysis of the human tumor S90 under the influence of titanocene dichloride confirms the antitumor activity of titanocene dichloride against colorectal carcinomas. There was a rapid and pronounced decrease of mitotic activity to about 10 % of control values (Fig. 2). Nuclear changes like chromatin condensation and formation of bizarre-shaped nuclei developed within 12 h after treatment with a single dose of titanocene dichloride (Fig. 4). Later on, an increased number of lipid droplets and lysosomes occurred in the cytoplasm of tumor cells and pointed to cytoplasmic degenera-

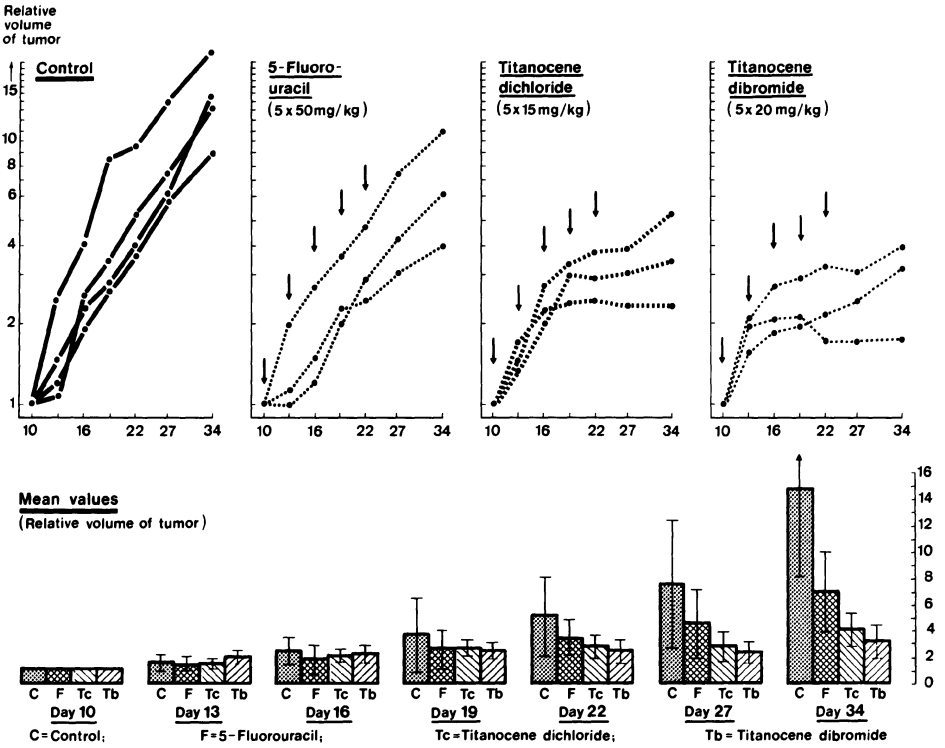


Fig. 1. Growth development of a human colon adenocarcinoma heterotransplanted into athymic mice under treatment with 5-fluorouracil, titanocene dichloride and titanocene dibromide, administered at equivalent sublethal doses on days 10, 13, 16, 19 and 22 after tumor transplantation. Upper part: growth curves of individual tumors; on abscissa, days after tumor implant on day 0; arrows indicate substance injections. Lower part: mean values of relative volume and standard deviations within control and treatment groups shown in the upper part.

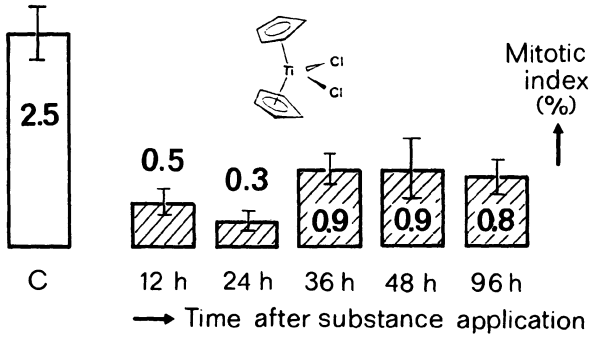


Fig. 2. Mitotic indices found in xenografted human colon adenocarcinoma S90 of control animals (C) and at different intervals after application of titanocene dichloride (1 x 40 mg/kg).

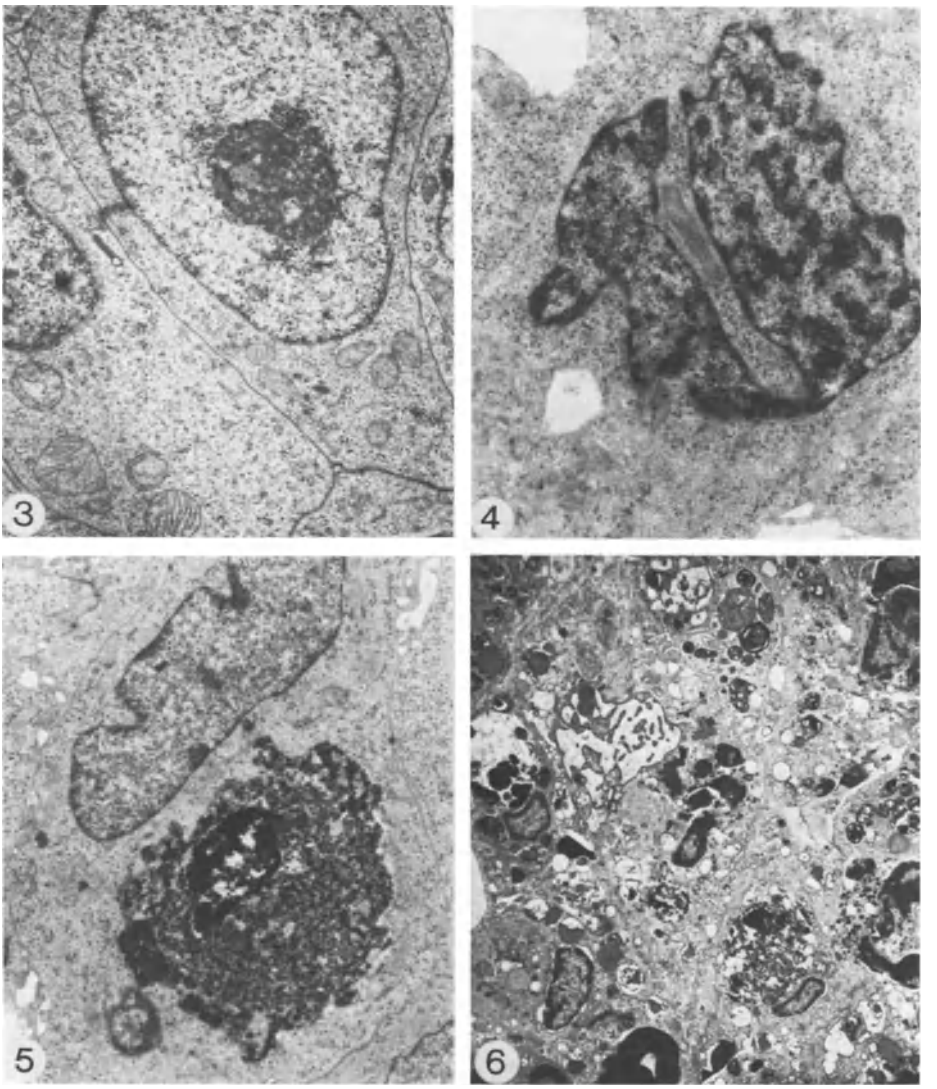


Fig. 3. Electron micrograph of untreated human colon adenocarcinoma S90. x 13.300
Fig. 4. Tumor cell 12 h after single injection of titanocene dichloride (40 mg/kg). Chromatin clumping and nuclear segmentation. x 12.700
Fig. 5. Part of a tumor cell 24 h after treatment with titanocene dichloride (1 x 40 mg/kg). Large cytoplasmic phagosome. x 7.700
Fig. 6. Human colon adenocarcinoma S90 24 h after threefold injections of titanocene dichloride. Tumor cells in all stages of degeneration. x 5.300

tion. Inclusion bodies often containing cellular debris appeared within tumor cells (Fig. 5). Intracytoplasmic viruses of type A and extracellular virus particles of type C were detectable 24 h after treatment and later. When threefold doses of titanocene dichloride were applied, the structural lesions which were induced were much more severe (Fig. 6) and remained irreversible during one week after the last substance injection.

TOXICOLOGIC FEATURES

The patterns of organ toxicity and teratogenicity provoked by treatment with titanocene dichloride were investigated in mice after application of single doses at the ED_{90} and LD_{10} levels.

Organ toxicity.

Neither the serum concentrations of creatinine and blood urea nitrogen nor the composition of the urine nor the morphologic appearance of the kidneys of animals treated with titanocene dichloride revealed any hints to nephrotoxicity induced by titanocene dichloride. No long-lasting elevations of blood retention values and no signs of glucosuria and proteinuria were detectable, no histologic and ultrastructural lesions of the kidneys were observed even after application of LD_{50} doses (18).

Whereas the kidneys apparently remained preserved, the serum levels of some liver enzymes such as GLDH, GOT and GPT increased by factors of 2-4 between 4 h and 2 days after single dose application (19). These functional impairments are indicative of an injury of the integrity of liver cells and were accompanied by the transient appearance of small lipid droplets in the cytoplasm of liver parenchyma cells and, at higher doses, by the occurrence of single cell necroses. It is known that similar pictures of fatty degeneration develop under the influence of hepatotoxic agents, e.g., aromatic hydrocarbons such as benzene and toluene. Because the cyclopentadienyl ring ligand also represents an aromatic hydrocarbon compound, it seems to be conceivable that it is actually responsible for the induction of dose-limiting hepatotoxicity after application of titanocene dichloride.

Other functional impairments concerned some endocrine glands

and the hormonal status of the animals and led to significant increases in the serum levels of glucagon and cortisol. Possibly, these increases were stimulated by the initial fall in serum glucose after drug administration (19).

Hematoxicity.

Regarding bone marrow function, there is apparently only slight depression of bone marrow function after application of titanocene dichloride. Neither the counts of mature and young erythrocytes nor the number of leukocytes were markedly depressed, only the count of circulating platelets in the peripheral blood decreased significantly 8 d after application of titanocene dichloride (20). Thus, thrombocytes apparently represent the only cells affected by titanocene dichloride. This pattern of mild hematoxicity is quite uncommon in comparison to other cytostatic agents which generally depress bone marrow function in a dose-limiting manner.

Embryotoxicity.

Another unusual finding was the lack of multiple malformations in new-borns after treatment of pregnant mice with titanocene dichloride during the sensitive phase of embryonal organogenesis. No gross deformities of the skeleton or of inner organs did occur when LD_{50} doses were applied. The only malformation which was found in 10-50 % of the fetuses treated during organogenesis was the dose-dependent appearance of cleft palate (21).

PHARMACOKINETIC STUDIES

The time-dependent organ distribution of titanium after single application of titanocene dichloride was determined by atomic absorption spectroscopy. Main accumulation of titanium was found in the liver and the intestine (Fig. 7) whereas the kidneys contained only half of the concentrations found in both other organs. This situation suggests titanium-containing metabolites being mainly eliminated via the bile and faeces and may be one of the reasons for the non-affection of the kidneys by titanocene dichloride. Whereas in diverse solid tumors growing subcutaneously considerable amounts of titanium were found, there was no apparent transfer of titanium-containing metabolites across the blood-brain-barrier. In analogy

to the latter result, titanium did also not enter the embryonal compartment and was not measurable in embryos or fetuses when pregnant mice were treated with titanocene dichloride on days 10, 12 or 14 of pregnancy.

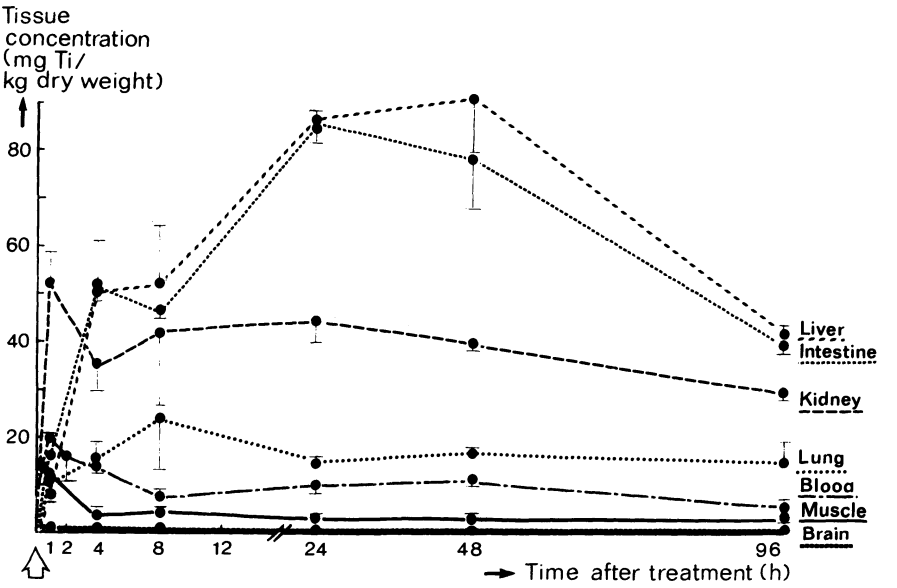


Fig. 7. Time-dependent organ distribution of titanium after single intraperitoneal treatment with titanocene dichloride (60 mg/kg) to NMRI mice at time 0. Control values ranging in all organs between 0.2 ± 0.05 and 0.6 ± 0.35.

CONCLUSIONS

The antitumor studies performed during the past years revealed antitumor potency for organometallic metallocene and ferricenium complexes. It must be clarified by future investigations if bis(cyclopentadienyl)titanium species, mono(cyclopentadienyl)titanium species or, perhaps, titanium or the cyclopentadienyl rings themselves are the biologically relevant moieties interacting with cellular molecules.

Metallocene complexes are characterized by spectra of activity

against experimental animal and human tumors which are not identical to those which are known from platinum complexes.

The toxic properties of metallocene complexes are fundamentally different from those of platinum compounds.

Thus, metallocene and metallicenium compounds do not represent analogues of antitumor platinum complexes, but must be considered as independent groups of antiproliferative agents.

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MODELLING AND SPECIATION OF METALS INVOLVED IN CARCINOGENESIS AND ITS TREATMENT

D.R. Williams

INTRODUCTION

In considering cancer chemotherapy, it is important to bear in mind Haddow's paradox that anything that can be used to treat a cancer can, under different circumstances, probably cause cancer, and also to pose the question as to how cancers are initiated (1).

Carcinogenesis may be triggered by heavy metal complexes which distort biochemical processes by forming chemical bonds to nucleic acids or, alternatively, by the metals or their complexes being isotopes which are radioactive emitting radiation which damages living cells (2).

There are three important events associated with these processes.

(i) The industrial revolution two centuries ago witnessed the mining of very many metals not normally present in our environment and which became an additional threat. However, simultaneously, man received new sources of energy in the form of fossil-based fuels and such energy led to improved standards of living.

(ii) The large number of pharmaceuticals which became available from the 1930s onwards meant that the diseases which had previously drastically abbreviated one's expected lifespan, could be overcome and life expectancies increased to about 70 years.

(iii) The commencement of the nuclear era in 1939-40 led to a whole new range of life-extending products based upon

a plentiful supply of energy. However, Three Mile Island, the Windscale fire, and the recent Chernobyl incidents have lucidly illustrated that the threat of cancer might well arise from nuclear sources.

Whether one considers metal complex anti-cancer compounds, organic carcinostats, or radionuclides, it is necessary to know details of the bioavailability and speciation of the elements before being able to comment upon the biochemical effects of the agents concerned.

THE SCOPE OF THE RESEARCHES

Our planet has 86 elements which are stable, approximately 20 of which are regarded as essential to man and approximately 10 which are toxic (3). There are, however, 108 elements known at this time, the increase over and above 86 being unstable elements or man-made elements such as the actinides. In addition, there are some of the stable 86 elements having unstable isotopes which emit radiation. 82% of the 108 elements are classified as metals and so a large amount of internal radiation damage is metal speciation dependent.

Since Werner's time, scientists have studied the metal-ligand complexes formed by these metals. Such studies can produce a much better understanding of, for example, the modus operandi of drugs, the bioavailability of metal complexes, and mechanisms of toxicity. The researches, however, have not been equally distributed across all the metal complexes in man and to which he is exposed.

Those metal ions which react with inorganic ligands or with organic ligands having electron donor groups have, in general, been deeply researched. Conversely, organometallic compounds (metal-carbon bonds) and metal ion colloid complexes have not been well characterised. Thus, we are not yet able to be confident about how such complexes react with human biochemistry.

Platinum falls into all of these categories - it complexes with inorganic ligands to produce compounds such as cisplatin, its organic ligand-metal ion chemistry (such as occurs with amino-acids and DNA), its ability to form organometallic compounds involving bonds to a carbon atom in a ligand and its existence in the colloidal state. The last two states it shares in common with a whole group of metals which are formed as nuclear fission products. Providing such agents are sufficiently robust to hydrolysis, they can follow the food chain into man and cause cancer by complexation or as a result of the radiation they emit. Thus, a knowledge of the speciation of the last two groups and of their radiobiology is highly desirable. Modelling is the preferred approach to both issues.

SPECIATION MODELS

When one deals with research topics as important as the side effects of new drugs, or decisions concerning radwaste disposal which have to hold true for, perhaps, half a million years - because of the long half-lives involved - it is essential (a) that mistakes are made 'on the drawing board' - albeit a computerised one rather than in reality, and (b) that any experiments designed to provide the data upon which the decisions are made are the most germane and the cost effective possible. Computer simulation is the only method of realising these objectives.

The field has been reviewed recently (4) and rests upon well tried physico-chemical equations, such as equilibrium constants, kinetic relationships etc., which have been established over the last century. These are combined into software modelling of a given scenario. The programs and models have to be well verified and validated, usually by international comparisons, and then run using the best databases available. The latter consist of thermodynamic, kinetic, geochemical data for all naturally occurring elements present in the disposal vault, the geo-

sphere and the biosphere. This data has to include all the actinides and fission products found in the radwaste. Elements such as platinum and palladium etc. ought to be included. Such database libraries typically contain 5-10 000 species. The prime objective is to calculate the radiation dose and/or risk to man from a given waste disposal plan. This is required before the licensing authorities grant permission for a route. Far more data is required before we can successfully model platinum as a fission product or as an anticancer agent.

CONCLUSIONS

Researchers into platinum and other metal coordination chemotherapies deserve the support of inorganic chemists who ought urgently to target their attentions towards providing data on these metal compounds so that one can model anticancer drugs in vivo and fission product emitting radiation in the biosphere.

Such speciation knowledge can help our understanding of bioavailabilities, of biomagnification by microbes, means of reducing toxicity of "inorganic" drugs, and the threat of radioactive metals produced as fission products.

Computer simulation is not a new field but the subject has taken a great leap forward with the advent of readily accessible data storage and user-friendly programs. It is particularly useful in tackling concentrations too low to be analyzed by known analytical techniques and also with radioactive metals having half-lives of many thousands of years and so beyond the timescale of any reasonable laboratory experiment.

ACKNOWLEDGEMENTS

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METAL COMPLEXES OF PLATINUM GROUP: THE PROMISING ANTITUMOR FEATURES OF *CIS*- DICHLOROTETRAKIS (DIMETHYLSULFOXIDE) Ru(II) [*cis*- $RuCl_2(Me_2SO)_4$] AND RELATED COMPLEXES

E. Alessio, W. Attia, M. Calligaris, S. Cauci, L. Dolzani, G. Mestroni, C. Monti-Bragadin, G. Nardin, F. Quadrifoglio, G. Sava, M. Tamaro and S. Zorzet

INTRODUCTION

After the discovery of the antineoplastic activity of cis-$PtCl_2(NH_3)_2$ (cisplatin, cis-DDP) (1), several Pt(II) derivatives have been synthesized and tested with the aim of developing new drugs having more pronounced antitumor activity and reduced host toxicity (2). Despite this effort, up to now only 3 out of more than 1000 platinum derivatives tested have reached the stage of clinical trials (3).

Although recent works show that Platinum derivatives can still reserve some good result (4,5), the interest in the screening of non-platinum complexes is growing.

Promising results have been obtained with complexes of Titanium (6), Rhodium (7), Iridium (8) and Ruthenium (9). Among the Ruthenium derivatives some Ru(III) complexes with nitrogen donor ligands (10) and a Ru(II) complex with dimethylsulfoxide, cis-$RuCl_2(Me_2SO)_4$ (11) deserve a particular interest.

We have shown that cis-$RuCl_2(Me_2SO)_4$ possesses mutagenic properties (12), a good antineoplastic activity against several murine metastasizing tumors (13) and interacts "in vitro" with DNA (14).

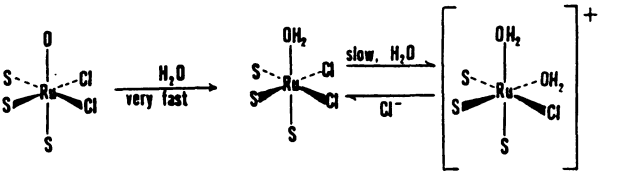
PART I: CHEMICAL PROPERTIES

New interesting perspectives were disclosed by our discovery that, in dimethylsulfoxide solution and at room temperature, cis-$RuCl_2(Me_2SO)_4$ can undergo a photo-induced isomerization to the up to now unknown trans isomer: trans-$RuCl_2(Me_2SO)_4$ (yield 80%).

The new complex has been characterized by means of usual spectroscopic techniques and its X-ray structure determined (15); it displays a crystallographic 4/m symmetry, with all the four dimethylsulfoxide molecules S-bonded to Ruthenium in the equatorial plane, like the already known dibromo derivative (16).

The promising results obtained with cis-$RuCl_2(Me_2SO)_4$ urged us to undertake a detailed study of the chemical behavior of the two isomers in water solutions, by means of conductivity and spectroscopic measurements (uv-vis, ^1H and ^{13}C NMR), with the aim to rationalize the results concerning their interaction with DNA as well as their microbiological and pharmacological properties.

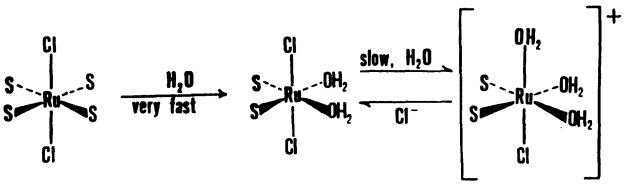
The chemical behavior of cis-$RuCl_2(Me_2SO)_4$ is summarized in Scheme 1:



Scheme 1. cis-$RuCl_2(Me_2SO)_4$ in aqueous solution (O = O-bonded Me_2SO, S = S-bonded Me_2SO)

Once dissolved , the complex immediately releases the O-bonded dimethylsulfoxide molecule, which is known to have a labile nature (11,17). This step is followed by the slow dissociation of a Cl^- anion to give the cationic species; the dissociation equilibrium is reached within 3 hours at 37 °C and, as expected, it is affected by the presence of free Cl^-. Two significant Cl^- concentrations have been tested, namely 3 mM and 150 mM, which represent the chloride concentration inside and outside the cell, respectively. While the equilibrium is not affected in 3 mM NaCl, it is completely inhibited in 150 mM NaCl.

trans-$RuCl_2(Me_2SO)_4$ shows a remarkably different behavior in water solution, summarized in Scheme 2:



Scheme 2. trans-$RuCl_2(Me_2SO)_4$ in aqueous solution

Once dissolved, the trans isomer immediately releases two dimethylsulfoxide molecules that, according to their strong trans effect, should be mutually cis. This step is again followed by the dissociation of a chloride anion to give the cationic species; the dissociation rate is

lower than in the case of the cis isomer, in agreement with the lower trans effect of chloride in comparison with dimethylsulfoxide. The equilibrium state is reached in more than 6 hours at 37 °C and again it is completely inhibited in phisiological solutions (150 mM NaCl).

Assuming that the coordinated water molecules have a labile nature, while cis-$RuCl_2(Me_2SO)_4$ in phisiological conditions has only one coordination site readily available, the trans isomer has immediately two sites in cis position. Accordingly a higher reactivity for the trans isomer is to be expected. Preliminary results are in agreement with the expected trend.

PART II: REACTION WITH DNA

When calf thymus DNA is incubated at 37°C in pseudo intracellular conditions (3mM NaCl, 1mM phosphate, pH 7.4) with both cis and trans isomers a covalent binding occurs. This reaction can be visualized with different spectroscopic techniques. The UV spectrum of reacted and ultrafiltered DNA shows an absorbance in the wavelength range beyond 300 nm, i.e. in the absorbance range of Ruthenium complexes. The ultrafiltration procedure, which consists of repeated washings with 0.5 M $NaClO_4$ and then with $2x10^{-4}$ M $NaClO_4$, insures that the retained Ruthenium is covalently and not simply electrostatically bound to DNA.

Fig. 1 shows a typical absorption spectrum of a Ruthenium-DNA complex compared with that of an unreacted DNA.

The rate of reaction of the two isomers with DNA is markedly different, being that of the trans isomer much faster. The different behaviour is shown in Fig. 2 where the amount of Ruthenium bound (measured with atomic absorption spectra) at 37°C and equimolar conditions for both isomers is plotted as a function of time. The trans isomer reaches a very high plateau (about 0.5 moles of Ruthenium per mol of phosphate) after about 10 hours of incubation, whereas the cis isomer, even after 4 days of incubation in the same conditions, does not exceed a value of 0.15 mol/mol.

This behaviour is in agreement with the dissociation schemes in aqueous solution of the two isomers: the trans possesses two coordination positions immediately available for binding, while the cis form has only one. When the reaction with DNA is carried out in the presence of 0.15 M Cl^- the trans isomer still reacts (r_b=0.09 at 20 h) whereas the reaction of the cis isomer is almost completely quenched (r_b=0.01 at 20 h).

The different pattern of reaction is paralleled by the modifications induced in the CD spectrum by both isomers. In fact, the cis isomer does not alter significantly the B-type spectrum of calf thymus DNA even after very long incubation times (Fig. 3), while the trans isomer, after reaction at low Cl^-, steadily decreases the intensity of both positive and negative CD bands of B-DNA, as already found in the case cis-DDP-DNA(18).

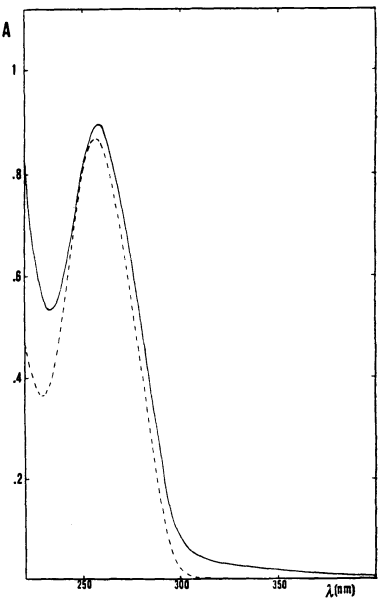


Fig.1. Absorption spectra of calf thymus DNA incubated for 7 days at 25°C with cis-$RuCl_2(Me_2SO)_4$ in equimolar conditions. Solvent: 3 mM NaCl, 1 mM phosphate pH 7.4 (———). For comparison the spectrum of untreated DNA is shown (-----).

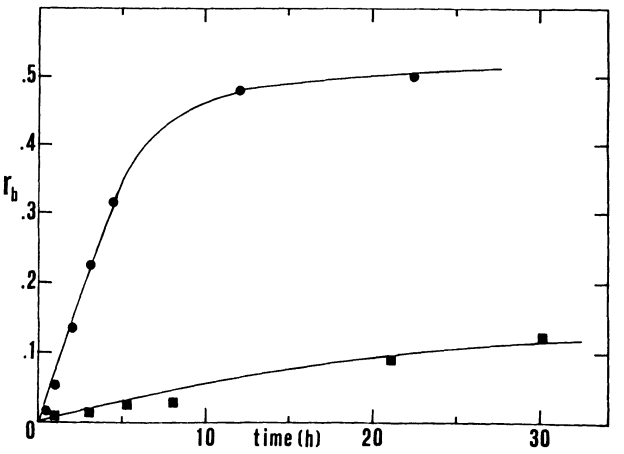


Fig. 2. Amount of Ruthenium bound determined by A.A.S. (●): trans-isomer; (□):cis-isomer.

The presence of Cl^- prevents this behaviour (Fig.3). The progressive modification with

time of the CD spectrum is highly indicative of a continuous change in the DNA structure.

When DNA, after reaction with either isomer, is heated, the termal behaviour is somewhat surprising when compared with that of DNA reacted with cis-DDP(19). The cis isomer of Ruthenium, in fact, produces a slight, but steady, increase in DNA melting temperature with the amount of bound metal which implies a progressive stabilization of the B conformation. On the other hand the trans isomer apparently increases the DNA melting temperature following the increase of the bound metal, but with a concomitant, clear decrease of the hyperchromic effect. When r_b is close to 0.5 the reacted DNA does not show any absorbance increase with temperature up to 90°C (in $2x10^{-4}$ M $NaClO_4$). This final condition is expressed also by the depressed CD spectrum shown in Fig. 3. This behaviour can be tentatively explained by the presence, in the DNA chain, of denatured regions, due to a particular type of Ruthenium binding (similar to those already demonstrated following cis-DDP binding (20)), and of regions of B-DNA (as those obtained after reaction with the cis isomer of Ruthenium) stabilized by another Ruthenium binding mechanism.

The preferred site of binding of both cis and trans isomers seems to be N7 of guanine residue, in agreement with what already found with cis- DDP and with the nucleophilic properties of that site (20). Such a suggestion comes from the reactions carried out with

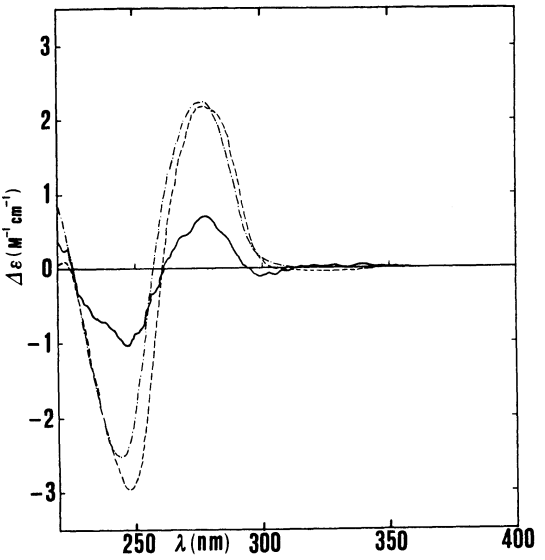


Fig.3. CD spectra of calf thymus DNA recorded (after filtration) in 0.2 mM $NaClO_4$ following incubation for 5 hours in 1 mM phosphate, pH 7.4 at 37°C, r_s=1 with (———) trans-isomer + 3 mM NaCl; (— · — · —) cis-isomer + 3 mM NaCl; (------) trans-isomer + 150 mM NaCl.

poly(dGdC). Fig. 4 shows the CD spectra, recorded at different times, of poly(dGdC) in the presence of equimolar amounts of cis-$RuCl_2(Me_2SO)_4$.

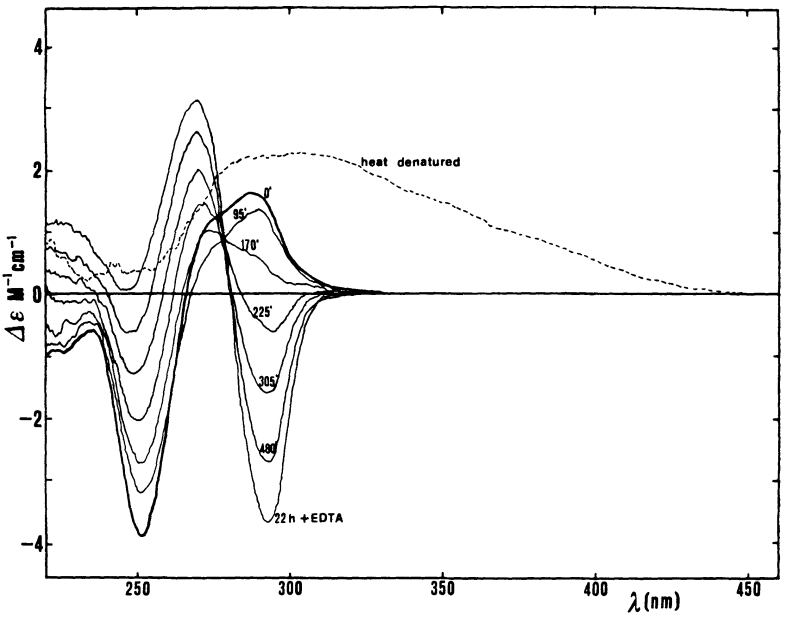


Fig. 4. CD spectra of poly(dGdC) dissolved in $2x10^{-4}$ M $NaClO_4$ in the presence of equimolar cis-$RuCl_2(Me_2SO)_4$ as a function of time at 37°C. The transition is complete after about 10 hours. The reacted polymer denatured at 90°C does not renature and shows a CD spectrum (-----) remarkably different from that corresponding to the unreacted, denatured polymer (not shown).

The data show a complete transition from B to Z structure of the polymer within 10 hours at 37°C. The transition is not due to trace amounts of di- or tri-valent cations and is not reversed by addition of EDTA (21). The finding can be explained by an income of a bulky group in the N7 position of guanine which, in turn, stabilizes the syn conformation of the N-glycosidic bond and, as consequence, the left-handed Z conformation (22). Again the rate at which the transformation occurs is much faster when the reaction is carried out with the trans isomer, as it appears from the comparison of the data shown in Figs. 5 and 6. Fig. 6 shows also that at longer times of incubation the Z structure induced by the trans isomer begins to collapse.

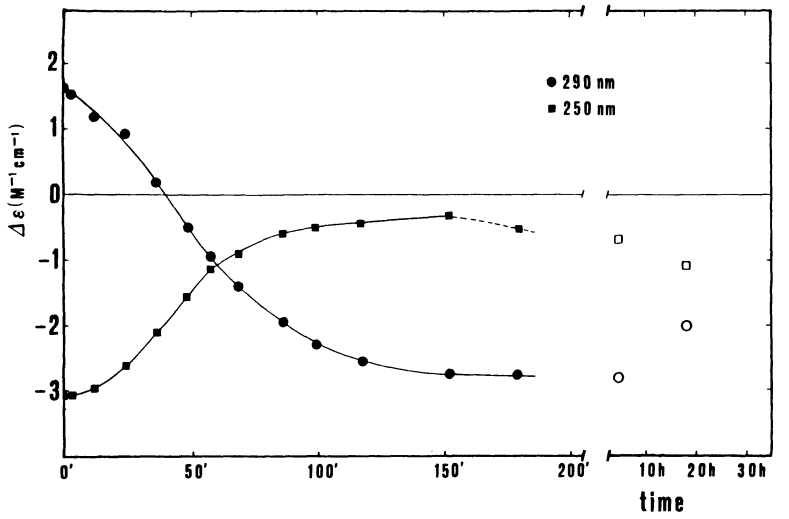


Fig. 5. Amplitude of CD bands, recorded at 250 and 290 nm, for poly(dGdC) (1.5×10^{-4} M) incubated with trans-$RuCl_2(Me_2SO)_4$ (molar ratio = 1) at 37°C in 2×10^{-4} M $NaClO_4$, as a function of time.

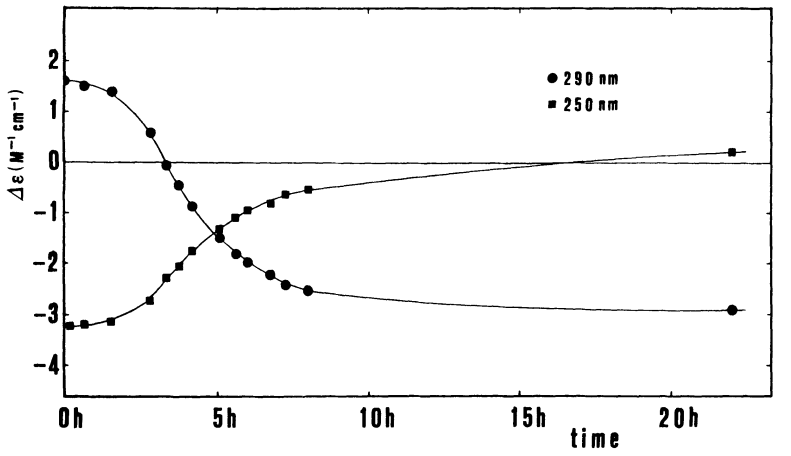


Fig.6. Amplitude of CD bands recorded at 250 and 290 nm for poly(dGdC) (1.5×10^{-4} M) incubated with cis-$RuCl_2(Me_2SO)_4$ (molar ratio = 1) at 37°C in 2×10^{-4} M $NaClO_4$ as a function of time.

PART III: EFFECTS ON BACTERIA

Bacterial tests offer a rapid and reliable tool to get some information about the mechanism of interaction of a drug with DNA. We decided therefore to study the effects of the cis and trans isomers of $RuCl_2(Me_2SO)_4$ on growth and mutation rate of two series of bacterial strains. These are *E. coli* and *S. typhimurium* derivatives with different DNA repair capability and carrying or not the pKM101 mutator plasmid. Their genetic characteristics are listed in Table 1.

Table 1. Genotype of the bacterial strains used

Strain	Genetic markers
Escherichia coli B (23)	
WP2	*trp*E
WP2uvrA	*trp*E *uvr*A
WP2recA	*trp*E *rec*A
WP2lexA	*trp*E *lex*A
WP2lexAuvrA	*trp*E *lex*A *uvr*A
TM 1	WP2/pKM101
TM 4	WP2uvrA/pKM101
Salmonella typhimurium (24)	
TA 92	*his*G46/pKM101
TA 98	*his*D3052, *rfa*, Δ *uvr*B/pKM101
TA 100	*his*G46, *rfa*, Δ *uvr*B/pKM101

Table 2 and Table 3 show the mutagenic activity of cis- and trans-$RuCl_2(Me_2SO)_4$, respectively. Mutagenesis tests were performed by the plate incorporation assay described by Ames (24). Drugs were dissolved in water and incorporated together with bacteria, in 2 ml of molten top agar, which were poured onto agar plates. Minimal agar media containing limiting amounts of nutrient broth for *E. coli* and of histidine for *S. typhimurium* were used. After a 48 hrs incubation at 37°C, colonies of bacteria reverted tp prototrophy were counted.
By comparing Tables 2 and 3, it is evident that the cis isomer of $RuCl_2(Me_2SO)_4$ has a stronger mutagenic activity than the trans one.

Table 2. Mutagenic activity of cis-$RuCl_2(Me_2SO)_4$ by plate incorporation assay.

Dose (µg/plate)	Bacterial strains: *Salmonella typhimurium* TA 92	TA 98	TA 100	WP2	*Escherichia coli* WP2uvrA	TM 1	TM 4
0	45	26	141	5	21	41	144
250							395
500					57	76	784
1000					91	119	1190
2000	91	80	310	7	152	192	1992
4000	130	120	423	15			
8000	157	167	547	19			

Values refer to the number of revertant colonies per plate and are the mean of 3 to 5 indipendent experiments.

Table 3. Mutagenic activity of trans-$RuCl_2(Me_2SO)_4$ by plate incorporation assay.

Dose (µg/plate)	Bacterial strains: *Salmonella typhimurium* TA 92	TA 98	TA 100	WP2	*Escherichia coli* WP2uvrA	TM 1	TM 4
0	40	15	114	8	13	21	91
250	40	16	213	9	18	18	120
500	40	18	258	5	16	22	131
1000	58	15	180	6	14	28	142
2000	83	14	tox	tox	27	tox	160
4000	tox	tox			tox		tox

Values refer to the number of revertant colonies and are the mean of 3 to 5 indipendent experiments.
Tox = toxic at the tested dose.

In fact, even at the higher tolerated doses of trans-$RuCl_2(Me_2SO)_4$ per plate, a doubling of the number of spontaneous revertants per plate is hardly attained, which is usually considered the minimal requirement for classifying a substance as a mutagen (25).

Table 4 shows the minimal concentrations of cis-$RuCl_2(Me_2SO)_4$ inhibiting growth of *E. coli* derivatives. When these experiments were performed using nutrient agar, strains lacking DNA repair enzymes demonstrated to be more sensitive to the inhibitory action of the drug than the wild parent strain, thus indicating that cis-$RuCl_2(Me_2SO)_4$ causes a repairable damage in DNA.

Table 4. Minimal Inhibitory Concentrations (MIC) of cis-$RuCl_2(Me_2SO)_4$ in different media.

Bacterial strain	MIC (µg/ml)	
	in nutrient agar	in minimal agar
WP2	>1000	>1000
WP2uvrA	1000	>1000
WP2lexA	1000	>1000
WP2recA	500	>1000
WP2lexAuvrA	500	>1000

To our surprise, when the minimal inhibitory concentrations (MIC) were measured in minimal agar plates, they resulted to be higher. As minimal agar plates are phosphate buffered ($[PO_4^{3-}]$ = 60 mM) we decided to investigate whether an interaction with inorganic phosphate (Pi) can be the cause of this behavior. For this purpose we assayed the MIC in nutrient broth ($[PO_4^{3-}]$ = 1.2 mM) additioned with increasing amounts of Pi. Table 5 shows that there is a direct relationship between Pi concentration and the MIC of cis-$RuCl_2(Me_2SO)_4$.

Table 5. Effect of inorganic phosphate concentrations (Pi) on MIC value of cis-$RuCl_2(Me_2SO)_4$ on WP2uvrA in nutrient broth.

$[PO_4^{3-}]$ mM	1.2	4.5	8.3	16	31
MIC (µg/ml)	1250	1750	2500	>2500	>2500

To assess if there is a direct interaction between cis-$RuCl_2(Me_2SO)_4$ and Pi, we mixed aqueous solutions of the two and let the mixture stand at room temperature in the dark before assaying mutagenic activity.

This was then tested by a modification of the usual mutagenicity test, according to which

a growing culture of WP2uvrA was exposed for short periods to cis-$RuCl_2(Me_2SO)_4$ in nutrient broth. Treated bacteria were then washed and plated for counting revertants and total survivors. Table 6 shows that preincubation of cis-$RuCl_2(Me_2SO)_4$ with Pi causes a lowering of the number of revertants induced by the drug in dependence on the preincubation period. It seems therefore likely that there is a direct interaction between cis-$RuCl_2(Me_2SO)_4$ and inorganic phosphate.

Table 6. Lowering of mutagenic activity of cis-$RuCl_2(Me_2SO)_4$ after preincubation with different Pi concentrations as a function of time.

cis-$RuCl_2(Me_2SO)_4$ dissolved in:	Preincubation time (hrs) 0	2	4	6	8	10	24
H_2O	22.6	19	16.5	15.5	14.6	14.1	10.0
16mM Pi	22.6	11	6.6	4	2.13	1.3	0.02
120mM Pi	22.6	1.33	0.8	0.03	0	0	0

cis-$RuCl_2(Me_2SO)_4$ 8 mM was preincubated (dark, room temperature) in water or in presence of different concentrations of Pi. After a 4 fold dilution, the mixture was assayed for mutagenic activity on WP2uvrA, through a modification of the usual mutagenicity assay (see text). Values refer to the number of induced revertants per 10^6 survivors.

PART IV: ANTITUMOR AND ANTIMETASTATIC PROPERTIES

The effects of equitoxic dosages of cis- and trans-$RuCl_2(Me_2SO)_4$ on primary tumor growth and on the formation of lung metastases were studied in mice bearing Lewis lung carcinoma. The tumor line, conventionally maintained in C57Bl female mice of 20 g (26), was propagated , for the reported experiments, in BD2F1 female hybrids (animal source: Charles River, Calco, Como, Italy). Tumor inocula were made by s.c. or i. m. implantations of tumor fragments prepared using 2-weeks old tumors finely minced with scissors and diluted with an equal volume of Dulbecco's saline. Alternatively, for i. v. tumor inoculation, a single cell suspension of tumor cells was prepared by filtering the minced tumor through a double layer of sterile gauze and centrifuging at 200xg for 10'. Tumor cell viability, after resuspension in an equal volume of Dulbecco's saline, was about 50% (Trypan blue exclusion test). Primary tumor growth, determined by caliper measurements, and the formation of lung metastases were evaluated according to already known procedures (13). The complexes tested were administered i. p. in volumes of 0.1 ml per 10 g body weight. Cisplatin (obtained by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI,

USA) and cis-$RuCl_2(Me_2SO)_4$ were dissolved in isotonic saline; trans-$RuCl_2(Me_2SO)_4$ and trans-$RuBr_2(Me_2SO)_4$ were dissolved by sonication for 5 sec in isotonic saline containing 1% sodium carboxymethylcellulose.

The treatment schedule chosen for studying the antitumor activity consists of daily i. p. injections for 14 consecutive days, starting 24 hr after s. c. tumor implantation. The dosage employed for each complex is the maximum tolerated dose and corresponds to the $LD_{0.05}$ extrapolated from the plots relating log_{dose} vs probit lethality. The toxicity of cis- and trans-$RuCl_2(Me_2SO)_4$ for normal BD2F1 mice, tested with the described treatment schedule, is reported in Table 7.

Table 7. Toxicity of cis- and trans-$RuCl_2(Me_2SO)_4$

Compound	LD_{50} (confidence limits)	$LD_{0.05}$
cis-$RuCl_2(Me_2SO)_4$	890 (952-832)	700
trans-$RuCl_2(Me_2SO)_4$	84 (112-58) *	37

Group of 5 BD2F1 female mice were treated i. p. daily for 14 consecutive days with at least two different dosages lower than that causing 100% deaths within one week from the end of treatment. Dose ratio between consecutive doses constant and was equal to 2. The determination of LD_{50} and the statistical analysis performed were made according to the method of Litchfield and Wilcoxon (27).

*: means statistically lower than that of the cis-$RuCl_2(Me_2SO)_4$ derivative, $p = 0.05$.

On a molar basis trans-$RuCl_2(Me_2SO)_4$ results significantly more toxic by a factor of ten than its cis isomer; the difference, owing also to different slopes, is much more evident for the $LD_{0.05}$ values resulting from the plots, which indicate a 20-fold lower dose of trans-$RuCl_2(Me_2SO)_4$ to be administered to tumored animals (37 vs 700 mg kg/day) as compared with cis-$RuCl_2(Me_2SO)_4$.

The results of the comparison of the antitumor activity of cis- and trans-$RuCl_2(Me_2SO)_4$ are reported in Table 8.

The treatment has no statistically significant effect on primary tumor growth whereas it reduces the number and weight of spontaneous lung metastasis formation to about 50% of the controls; the effect of trans-$RuCl_2(Me_2SO)_4$ on metastasis weight is slightly superior than that of the cis isomer (inhibition to about 30% of the controls) and is of the same order of magnitude of that obtained with an equitoxic dosage of cisplatin. The absence of significant

Table 8. Comparison of the antineoplastic action of cis- and trans-$RuCl_2(Me_2SO)_4$ in mice bearing Lewis lung carcinoma.

Compound	Dose (mg/kg/day)	Primary tumor weight (mg)[a]		Lung metastases[b]			
				number		weight (mg)	
		mean±S.E.	%I	means±S.E.	%I	mean±S.E.	%I
Controls	—	2513±275	–	41±3	–	216±3	–
cis-$RuCl_2(Me_2SO)_4$	700	1809±352	28	22±2*	46	104±17*	52
trans-$RuCl_2(Me_2SO)_4$	37	1960±226	22	18±2*	57	62±11*	71
cis-$PtCl_2(NH_3)_2$	0.52	1608±402	36	14±4*	67	47±19*	78

a: measured on day 14 from tumor implantation

b: measured on day 21 from tumor implantation.

%I: means percent inhibition compared to the value obtained in control group.

Groups of 8 BD2F1 mice, inoculated s. c. with 100 mm^3 of Lewis lung carcinoma fragments on day 0, were given i.p. on days 1-14 the reported compounds.

*: means statistically different from the corresponding value of control group, Student-Newmann-Keuls test (28), p = 0.05.

activity on primary tumor growth is in agreement with separate observations which indicate a strict dependence of the antitumor action on the mass of primary tumor, showing a statistically significant inhibition of primary tumor growth only for tumors which, 2 weeks after implantation, do not exceed 1 g per animal.

The antimetastatic effect of cis-$RuCl_2(Me_2SO)_4$ and that of the trans-dibromo and trans-dichloro isomers were compared to that of cisplatin in mice with lung tumor colonies artificially induced by i. v. implantation of Lewis lung carcinoma cells (Table 9). Data reported in Table 9 show the inactivity of cis-$RuCl_2(Me_2SO)_4$.

Indeed, the complex could result inactive because of the inappropriate dose-level employed in the experiment: the low toxicity of this complex allows the use of dosages higher than 800 mg/kg/day for 5 consecutive days which needs further investigation. Conversely, the dosage used for the trans isomer is slightly toxic for the host, being responsible for toxic

Table 9. Effects of cis- and trans-$RuCl_2(Me_2SO)_4$ on the growth of artificial lung metastases of Lewis lung carcinoma.

Compound	Dose (mg/kg/day)	Toxic deaths	Lung metastases[a] number means±S.E.	%I	weight (mg) mean±S.E.	%I	animals free[b]
Controls	—	0/8	9.2±1.1	—	169±27	—	0/8
cis-$RuCl_2(Me_2SO)_4$	800	0/8	10.5±3.3	-14	133±32	21	0/8
trans-$RuBr_2(Me_2SO)_4$	150	3/8	7.3±1.4	21	38±2*	78	0/5
trans-$RuCl_2(Me_2SO)_4$	150	3/8	5	46	15	91	4/5
cis-$PtCl_2(NH_3)_2$	2.5	0/8	4.0±0.6*	56	7±1*	96	3/8

a: measured on day 14 from i. v. tumor implantation.

b: animals free of macroscopically detectable lung colonies.

Groups of 8 BD2F1 mice, receiving i.v. $2.5x10^5$ Lewis lung carcinoma cells (artificial metastasis induction) on day 0, were given i.p. the compounds indicated on days 1-5.

*: means statistically different from the corresponding value of the control group, Student-Newmann-Keuls test (28), p = 0.05.

deaths within the treated groups. Nevertheless, these complexes show that the potency of the trans-dichloro isomer is higher than that of the trans-dibromo one, indicating the influence of the alogen ligand on the antitumor effect. In this experiment cisplatin, at a dosage considered maximum tolerated for the length of the treatment schedule employed, inhibits metastasis formation to a pronounced degree, evidencing a clear superiority over the Ruthenium tested derivatives.

The postsurgical treatment with cis-$RuCl_2(Me_2SO)_4$ and trans-$RuCl_2(Me_2SO)_4$ causes, in mice subjected to surgical ablation of the tumored leg under conventional procedures (13), a statistically significant prolongation of the survival time of the treated animals (Figure 7).

The effects of cisplatin are much less impressive and the prolongation of the life-span of

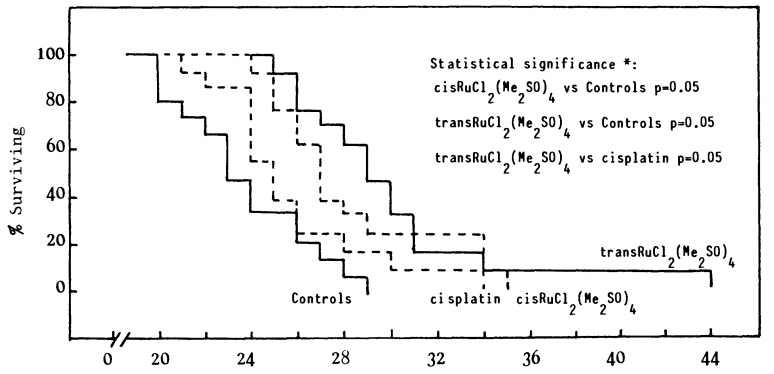


Figure 7. Effects of cis-$RuCl_2(Me_2SO)_4$, trans-$RuCl_2(Me_2SO)_4$ and cisplatin on the survival time of mice bearing Lewis lung carcinoma and undergoing surgical removal of primary tumor. Groups of 12 BD2F1 mice (15 controls) were randomly prepared after surgical amputation of the primary tumor (average weight of the tumor removed: 3.4±0.1 g), inoculated i.m. into the calf of the left hand leg 10 days before. The drugs were given daily on days 1-10 after surgery using the same dosages reported in Table 8 (0.52 mg/kg/day for cisplatin).
*: determined by the method of Student-Newmann-Keuls test (28), p = 0.05.

the treated animals is not statistically different from that of the controls. The lack of significant activity of cisplatin can be attributed to the daily dose administered which, because of the length of treatment, is low in order to avoid toxicity for the host in mice which underwent surgical intervention and anesthesia. The treatment with the higher dose (2.5 mg/kg/day given i. p. for 5 days after surgery) causes a statistically significant prolongation of the life-span of the treated hosts, qualitatively intermediate between those of cis- and trans-$RuCl_2(Me_2SO)_4$ (unreported results). It thus seems that, indipendently from the effects on metastasis formation, as evaluated after sacrifice of the host, which indicate cisplatin the most potent compound, the therapeutic potential of Ruthenium complexes is significant and comparable to that of cisplatin when the survival time of the host is considered.

CONCLUSIONS

The main conclusions which can be drawn from this work are the following:

a) the mutagenic activity of cis-$RuCl_2(Me_2SO)_4$ suggests that DNA is the preferred target in vivo. The in vitro experiments show that cis isomer easily reacts with DNA.

b) despite of its in vitro higher rate of reaction with DNA the trans-isomer exhibits a lower mutagenic activity in the Ames test. This effect could be related to the higher toxicity of trans-isomer for the bacterial strains used, which enables the compound to reach intracellular concentrations capable of mutagenic effects. This, in turn, suggests, due to its higher

reactivity, trans-$RuCl_2(Me_2SO)_4$ could react with other cellular components in addition to DNA. Furthermore these reactions might modify the redox potential of the complex and consequently produce a more stable, inactive Ru(III) compound via oxidation. On this light it seems important to evaluate the mutagenic activity in nitrogen saturated atmosphere.

c) the higher in vitro reactivity of trans-$RuCl_2(Me_2SO)_4$ correlates with a higher in vivo toxicity for tumor bearing hosts, but not with the antineoplastic activity. The comparison of the antitumor and antimetastatic activities shows only a slightly better antimetastatic effect for trans-$RuCl_2(Me_2SO)_4$ as compared to cis-isomer. The use of equitoxic dosages of cis- and trans-$RuCl_2(Me_2SO)_4$ and of cisplatin evidentiates that the therapeutic potential of Ruthenium derivatives is higher than that of cisplatin.

d) the data obtained with the two Ruthenium isomers indicate the biological potential of these compounds and suggest the need for an extension of the study to other cis and trans complexes with different ligands.

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NMR STUDY OF PROPERTIES IN SOLUTION OF PLATINUM(II) AMMINE-AQUA COMPLEXES, INCLUDING DETERMINATION OF ACID DISSOCIATION CONSTANTS

T.G. Appleton, J.R. Hall and S.F. Ralph

INTRODUCTION

In view of the interest in cis-$Pt(NH_3)_2Cl_2$ and its derivatives in recent years, it is remarkable that there are, to our knowledge, no values from reliable experimental determinations in the literature for the acid dissociation constants of such simple species as cis-$Pt(NH_3)_2(H_2O)_2^{2+}$ (I) and cis-$Pt(NH_3)_2Cl(H_2O)^+$ (II). Grinberg and Ryabchikov (1) reported that, although trans-$Pt(NH_3)_2(H_2O)_2^{2+}$ with NaOH gave a simple titration curve with two distinct "breaks" corresponding to the two deprotonation steps, the cis isomer (I) gave a much more complex curve which they claimed could give only an average of the two acid dissociation constants. If the data presented by Jensen (2) are plotted, it is clear that he, too, obtained a complex curve, but he nevertheless analysed these data to give values (at 20°C) of pK_{a1} = 5.56 and pK_{a2} = 7.32. Perumareddi and Adamson (3) gave no details of their procedure, but reported pK_{a1} = 5.63 and pK_{a2} = 9.25. We now know (4,5) that in solutions containing moderate concentrations of cis-$Pt(NH_3)_2(OH)(H_2O)^+$ there is rapid formation of hydroxo-bridged oligomers $[Pt(NH_3)_2(\mu\text{-}OH)]_n^{n+}$ (n = 2,3). Attempts to determine the acid dissociation constants which do not take this into account cannot be expected to produce reliable results, especially for pK_{a2}. The situation is even more complex for cis-$Pt(NH_3)_2Cl(H_2O)^+$ (II), since this complex is always present in solution together with (I) and cis-$Pt(NH_3)_2Cl_2$. From comparisons with other complexes, Martin (6) estimated that pK_a would be near 6.3.

If ^{15}N substituted ammine ligands are used, NMR spectroscopy is

a powerful means for characterizing complexes in solution (4,5,7-9). The ^{15}N chemical shift, δ_N, and the ^{195}Pt-^{15}N coupling constant (^{195}Pt, I = ½, 34% abundance; ^{15}N, I = ½) are both sensitive to the ligand trans to ammine. It occurred to us that acid dissociation constants could be obtained by measuring the change in one of these parameters with pH. Even if the species of interest is only a minor component in a complex mixture, this method can be used, as long as the appropriate peaks can be identified in the ^{15}N spectrum of the solution.

RESULTS

$Pt(NH_3)_3(H_2O)^{2+}$ (III)

This complex was studied first, because, although ^{15}N and ^{195}Pt NMR showed that the equilibrium [1] was set up, this was sufficiently slow to allow the pK_a for (III) to be determined easily both by NMR and conventional potentiometric methods.

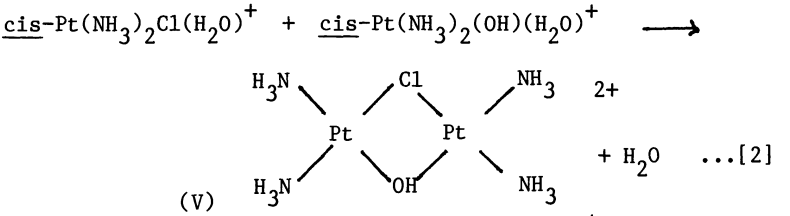
$$\underset{\text{(III)}}{Pt(NH_3)_3(H_2O)^{2+}} + Pt(NH_3)_3(OH)^{+} \rightleftharpoons \underset{\text{(IV)}}{\{Pt(NH_3)_3\}_2(\mu\text{-}OH)^{3+}} + H_2O \quad \ldots[1]$$

^{15}N NMR spectra at each pH gave the chemical shifts for ammine nitrogen cis and trans to water/hydroxide, and Pt - N coupling constants for these N-atoms. Chemical shift parameters were more convenient to use, as NMR accumulation times could be shorter than when the weaker "satellite" peaks had to be observed. ^{N} trans to water/hydroxide was, as expected, more sensitive to the deprotonation, and would therefore be used in preference to ^{N} cis to water/hydroxide. For (III), all four NMR parameters were plotted against pH, and the value of pK_a determined as the pH corresponding to the inflection point on the curve. Each plot gave the same value for pK_a at 25°C, 6.40 ± 0.1, which was in agreement with the value from our potentiometric determination.

cis-$Pt(NH_3)_2Cl(H_2O)^{+}$ (II)

An aqueous solution containing (II) was prepared by addition of one mole equivalent of NaCl to an aqueous solution of cis-$Pt(^{15}NH_3)_2(H_2O)_2^{2+}$ (I). Although (II) was the major species in these solutions, some cis-$Pt(^{15}NH_3)_2Cl_2$ also formed (most precipitating), and unreacted (I) was also present. When NaOH solution was

added to increase the pH to the vicinity of 7, NMR spectra showed that a new species was formed, which was formulated as (V) (reaction [2])



Significant amounts of $[Pt(NH_3)_2(\mu\text{-}OH)]_n^{n+}$ were also formed, and, on long standing at pH 7 became the dominant species. If the NMR measurements were carried out as quickly as possible, peaks due to the monomeric chloro complex remained detectable. Fig. 1 shows a plot of δ_N for ammine trans to water/hydroxide against pH, which gave a value for pKa at 25°C 6.70 ± 0.10.

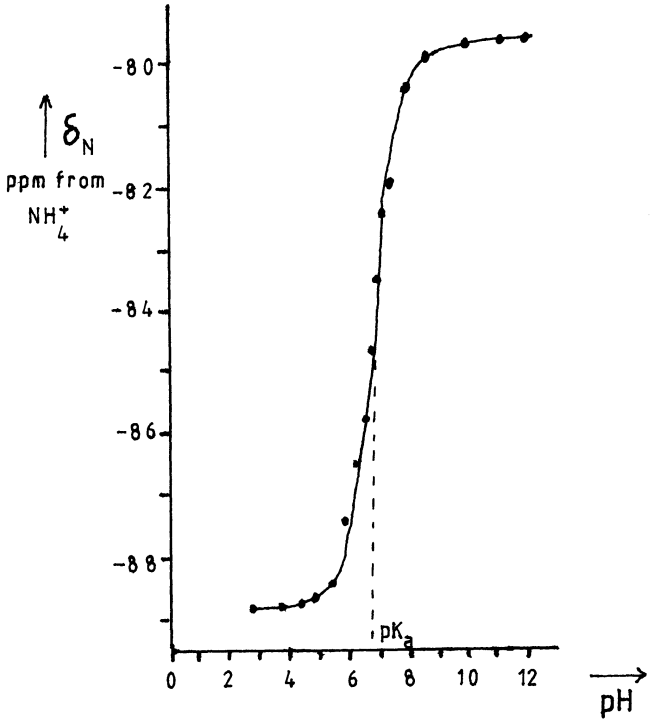


Fig. 1. Plot of ^{15}N chemical shift for ammine trans to water/hydroxide in cis-$Pt(NH_3)_2Cl(H_2O)^+$/$Pt(NH_3)_2Cl(OH)$ against pH at 25°C.

Cis-$Pt(NH_3)_2(H_2O)_2^{2+}$ (I)

The dimerization of cis-$Pt(NH_3)_2(OH)(H_2O)^+$ was so fast that, even using the NMR method, it was necessary to carry out the measurements at 5°C to reduce this rate. The plot of $_N$ against pH did not show two distinct "breaks", but the shape of the curve was in excellent agreement with the "theoretical" curve calculated using the expression [3].

$$\frac{[H^+]}{K_{a1}}(\delta_N - \delta_{A_2}) + \frac{K_{a2}(\delta_N - \delta_{H_2})}{[H^+]} = \delta_{AH} - \delta_N \quad \ldots[3]$$

where δ_N is the measured ^{15}N chemical shift at the pH of measurement

δ_{A_2} is the ^{15}N chemical shift of cis-$Pt(^{15}NH_3)_2(H_2O)_2^{2+}$

δ_{H_2} is the ^{15}N chemical shift of cis-$Pt(^{15}NH_3)_2(OH)_2$

δ_{AH} is the (time-averaged) ^{15}N chemical shift of cis-$Pt(^{15}NH_3)_2(OH)(H_2O)^+$ (estimated using empirical trans influence parameters (9)).

From a comparison of the experimental curve with the "theoretical" curves generated from different values of pK_{a1} and pK_{a2}, the values of these constants were determined as 5.95 ± 0.10 and 7.85 ± 0.10 respectively (5°C).

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STUDIES ON RUTHENIUM NITROIMIDAZOLES COMPLEXES AS RADIOSENSITIZERS

P.K.L. Chan, K.A. Skov, B.R. James and N.P. Farrell

Local control of tumours by radiotherapy may fail due to the presence of hypoxic cells in tumours. Nitroimidazoles enchance killing of these resistant cells with ionizing radiation, presumably due to their electron affinity which permits electron abstraction from irradiated DNA to increase cell kill (1). However, dose-limiting side-effects have prevented the attainment of maximum sensitization by nitroimidazoles in the clinic. A desirable aim is to decrease the overall concentration of radiosensitizer (to decrease side-effects), while retaining a high drug concentration at the target of ionizing radiation, the DNA. The attachment of a radiosensitizer to metals that bind to DNA may serve this purpose (2). The successful chemotherapeutic drug, cisplatin and analogues have shown moderate radiosensitizing effects (3) possibly due to their DNA binding rather than by electron affinity. As a consequence of binding to DNA, the metal could act as a carrier of radiosensitizer to the target while maintaining its own radiosensitizing effect.

We have been assessing some ruthenium (Ru) complexes of nitroimidazoles for radiosensitizing ability. Approaches using Ru as antineoplastic agents have been related to its DNA binding properties. Additional advantages of Ru are the availability of both the Ru(II) and Ru(III) oxidation states under physiological conditions and the general substitution inertness of these ions when coordinated to nitrogen ligands (4). A series of Ru complexes $RuCl_2(dmso)_2L_n$ (L is a 2-, 4- or 5- nitroimidazole and n=1,2) have

been synthesized from cis-$RuCl_2(dmso)_4$, their radiosensitizing abilities and toxicities in Chinese hamster ovary cells and their DNA binding properties have been examined.

Our results on radiosensitization by a series of Ru complexes, and by free ligands, are shown in Table 1. The sensitizer enhancement ratio (SER) is calculated as the ratio of x-ray doses between nitrogen and chemicals at a surviving fraction of 0.01. The toxicity in Table 1 refers to the plating efficiency (P.E.) after 4 h of incubation at 37°C. Control P.E. was 0.81. It is interesting that metallation decreases the toxicity of some of these nitroimidazoles.

Table 1: Toxicity and SER values of the Ru compounds compared with nitroimidazole ligands in CHO cells (data from (5) and (6))

	Free Ligands		Ru Complexes	
	Toxicity	SER	Toxicity	SER
	(400 μM)		(200 μM)	
Misonidazole	0.75	1.3	0.77	1.4
2-nitroimidazole	0.0032	1.3	0.50	1.4
4-nitroimidazole	0.051	1.2	0.10	1.6
metronidazole	0.80	1.1	0.79	1.2
	(200 μM)		(100 μM)	
RSU-1170	0.037	1.1	0.15	1.3
RSU-3083	0.036	1.2	0.12	1.2
RSU-3100	0.0042*	1.2*	0.12	1.1
RSU-3159	0.0027*	1.3*	0.16	1.3

*100 μM (see 6)

Ru complexes of misonidazole (a 2-nitroimidazole) and its analogues were unstable in aqueous solution. Thus, they did not give significant increase in SER value when compared with the corresponding free ligands. The complex of the 5-nitroimidazole,

metronidazole, also dissociates. We have reported that $RuCl_2(dmso)_2(4\text{-nitroimidazole})_2$, Ru-4-nitro, gave a good enhancement ratio (1.6 at 200 μM and 1.4 at 100 μM) compared with its free ligand (1.2 at 400 μM)(5). However, the N-substituted-4-nitroimidazole complexes of Ru were not better than ligand alone (6). The structures of the N-substituted-4-nitroimidazoles are given in (6). No radiosensitizing enhancement or protection was observed at 400 μM DMSO and 100 μM $RuCl_2(DMSO)_4$ as control experiments.

A question has been raised as to whether these metal radiosensitizer complexes exert a genotoxic effect on normal cells. We have studied the genotoxic effect of Ru-4-nitro by a chromosome aberrations in vitro assay (7). The results showed that the % metaphases with chromatid aberrations following exposure to Ru-4-nitro was much less than that elicited by cisplatin and similar to that of misonidazole.

Table 2: Clastogenic Activity of Ru-4-nitro, misonidazole, 4-nitroimidazole and cisplatin in CHO cells (data from (7))

Average % metaphase plates with chromatid aberrations							
Ru-4-nitro (mM)	-S9*	misonidazole (mM)	-S9	4-nitroimidazole (mM)	-S9	cisplatin (μM)	-S9
10.0	31±3	10.0	26±3	10.0	16±3	50.0	T+
6.0	26±2	6.0	22±2	6.0	13±2	30.0	T+
2.0	19±1	2.0	16±2	2.0	10±2	10.0	MI‡
1.0	14±2	1.0	12±2	1.0	7±2	8.0	61±5
0.4	9±2	0.4	8±2	0.4	4±1	4.0	45±4
0.2	4±1	0.2	5±1	0.2	1±1	2.0	30±5

* Addition of S9 did not increase the extent of damage
+ T, toxic
‡ MI, mitotic inhibition: fewer than 40 diploid metaphases per plate observed.

Studies to determine why the Ru complex of 4-nitroimidazole is a better sensitizer than the N-substituted-4-nitroimidazole complexes of Ru are being carried out. At present, there are several possible reasons for this observation (Table 3):

Table 3: Reduction potentials, DNA binding properties and Cl^- detection of Ru complexes (data from (5) and (6))

Nitromidazole Ligand	$E_{½}$ (mV) L	Ru-L_n	DNA binding	Cl^- detected in aqueous solution
Misonidazole	-400	-385	-	-
2-nitroimidazole	-445	-435	-	-
4-nitroimidazole	-685	-615	+	+
RSU-1170	-410	-390	-	-
RSU-3083	-560	-540	-	-
RSU-3100	-470	-455	-	-
RSU-3159	-370	-360	-	-

(i) the attachment of Ru to the 4-nitroimidazole ligands increases the reduction potential of the free ligands, i.e. the ligand centers are more easily reduced when coordinated to Ru. In fact, Ru-4-nitro showed the largest increase in reduction potential ($\sim$ 70mV)(5);

(ii) Ru-4-nitro inhibited the endonuclease activity of BamHl on plasmid DNA, taken to be an indication of binding to DNA. The N-substituted-4-nitroimidazole complexes did not inhibit the restriction enzyme.(6)

(iii) There was no Cl^- release from the N-substituted-4-nitroimidazole Ru complexes in aqueous solution whereas Cl^- is released from Ru-4-nitro. Hydrolysis may be required to generate an active reagent, as demonstrated for other Ru(II) species (8) and cisplatin.

(iv) Ru-4-nitro has an N-H group on the imidazole ring which is absent in the substituted analogues, and which may be required as a hydrogen-bond donor to stabilize DNA binding of the complex (9).

The isolation of the aquated active-species of the active complex is being attempted. New 4-nitroimidazole complexes of Ru with different sulphoxide ligands are being synthesized to increase the stability of these complexes.

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PLATINUM(II) COMPLEXES WITH ALKYLAMINES. CHARACTERIZATION AND *IN VITRO* CYTOSTATIC ACTIVITY

V. Cherchi, G. Faraglia, L. Sindellari, G. Voltarel, S. Sitran, A. Furlani, L. Ravalico and V. Scarcia

INTRODUCTION

In an attempt to determine the role of inert and labile groups in the antitumour activity and toxicity of platinum - amine complexes, J. P. Macquet observed that modifications in the inert groups may modulate the activity against tumour cells, whereas changes in the leaving groups may affect the "in vivo" toxicity (1). In the last few years we carried out a systematic study on the synthesis of pure samples of neutral and ionic platinum complexes with straight chain amines in order to appreciate the cytostatic activity changes with the chain length increase. In previous papers we reported the synthesis and characterization of the complexes having generalformulae cis- and trans-$|PtL_2X_2|$, $|PtL_3X|X$ and $|PtL_4|X_2$, where X = halide and L = propan-1-amine (Pra) and hexan-1-amine (Hea) (2-5).

This note reports the synthesis of the analogous derivatives with butan-1-amine (Bua), pentan-1-amine (Pea) and heptan-1-amine (Hpa). The complexes have been characterized by infrared and ^1H n. m. r. spectroscopy and by thermogravimetric data (TG and DTA). Moreover preliminary tests of "in vitro" cytostatic activity against KB tumour cells have been carried out.

MATERIALS AND METHODS

The reagents used were $PtCl_2$ (Fluka), $K_2|PtCl_4|$ (Johnson Matthey), butan-1-amine (Bua, C. Erba), pentan-1-amine (Pea) and heptan-1-amine (Hpa, Fluka).

Preparation of the complexes

cis-$|PtL_2Cl_2|$

The compound cis-$|PtBua_2Cl_2|$ was prepared by reaction of $PtCl_2$ and Bua (molar ratio 1 : 2.2) in benzene. The suspension changed overnight with stirring into a dull white solid, which was filtered, washed with benzene and dried "in vacuo". M.p., 188-9°C. The complex cis-$|PtHpa_2Cl_2|$ was prepared analogously whereas the synthesis of cis-$|PtPea_2Cl_2|$ was performed in MeOH (M.p., 179-80°C). The complexes are insoluble in H_2O, acetone and benzene. They are slightly soluble in MeOH.

trans-$|PtL_2Cl_2|$

The complex trans-$|PtBua_2Cl_2|$ was prepared by refluxing (30 min) a suspension of $PtCl_2$ in a p-xylene solution of Bua (molar ratio 1 : 2.2). A yellow solution was obtained which separated yellow plates on cooling. The crystals were filtered and washed with n-hexane. M.p., 148-50°C. The complex trans-$|PtHpa_2Cl_2|$ was prepared by the same method whereas trans-$|PtPea_2Cl_2|$ was obtained by reflux of a toluene solution of $PtCl_2$ and amine (molar ratio 1 : 2.2; 2 h). The yellow solution was filtered from a small undissolved residue and then treated with n-hexane. The yellow crystals were filtered and washed with n-hexane. M.p., 126-7°C. The complexes are soluble in MeOH, acetone and benzene and insoluble in H_2O.

$|PtL_3Cl|Cl$

The compound $|PtPea_3Cl|Cl$ was obtained by stirring overnight a suspension of $PtCl_2$ in a benzene solution of Pea (molar ratio 1 : 4). The reaction went on in heterogeneous phase yielding the white solid, which was filtered and washed with n-hexane. M.p.,

178-81°C. The complex $|PtHpa_3Cl|Cl$ was prepared by the same procedure. The complexes are insoluble in benzene, acetone and H_2O and slightly soluble in MeOH.

$|PtPea_4|Cl_2$

An aqueous solution containing $K_2|PtCl_4|$ and Pea (molar ratio 1 : 6) was heated until colourless (80°C, 30 min). White crystals separated on cooling, which were washed with H_2O and dried " in vacuo ". M.p., 116-9°C. The compound is insoluble in H_2O, acetone and benzene and slightly soluble in MeOH.

The analytical data (C, H, N) of the complexes reported above agree well with the theoretical values. The parent complexes with propan-1-amine (Pra) and hexan-1-amine (Hea) were prepared as reported in references 2 and 3.

Measurements

The i. r. spectra were recorded by using a Perkin Elmer 580B spectrophotometer (4000 - 250 cm^{-1}) as Nujol mulls between CsI discs. The ^1H n. m. r. spectra were obtained using a Jeol FX 90Q spectrometer. The TG, DTG and DTA curves in dinitrogen (flux rate, 250 $cm^3 min^{-1}$; heating rate, 5°C min^{-1}) were recorded on a Netzsch STA 429 thermoanalytical instrument.

"In vitro" cytostatic activity

The substances have been tested following the method reported in ref. 6. In brief Minimal Eagle's Medium (MEM) supplemented with 10 % calf serum was used. 10^5 KB cells, a line derived from a human epidermoid carcinoma of the mouth, were incubated at 37°C in Leighton tubes. After 24 h the cells were attached to the glass and the compound to be tested, suspended in sterile saline, was then added. Incubation was carried out at 37°C for 72 h. Cell growth was estimated by counting in the Bürker chambre the viable cells detached from the glass wall with trypsin. The cytostatic activity was expressed as concentration of the compound in mol l^{-1} at which the cells showed

a 50 % inhibition of growth in relation to the control values (ID_{50}).

The statistical evaluation of the results was done by the Student "t" test.

RESULTS

Except for $|PtPea_4|Cl_2$, the complexes were prepared and purified in organic media. Due to the general insolubility in water, the cytotoxicity tests were carried out on sonicated suspensions.

Table 1. Infrared data (cm^{-1}).

Compound	ν(NH)	$\delta(NH_2)$	ν(Pt-Cl)	ν(Pt-N)
cis-$\|PtBua_2Cl_2\|$	3220sbr, 3125m	1578s	320s	270wbr
trans-$\|PtBua_2Cl_2\|$	3258m, 3215m, 3148mw	1570sbr	320s	260wbr
cis-$\|PtPea_2Cl_2\|$	(3223,3210,3190)wbr, 3125m	1577s	318s	278wbr
trans-$\|PtPea_2Cl_2\|$	3250m, 3230m, 3155mw	1595s	338s	263wbr
$\|PtPea_3Cl\|Cl$	3225sh, 3170sbr, 3108m	1578m	340m	290sbr
$\|PtPea_4\|Cl_2$	3100sbr	1616m 1590sh		295sbr
cis-$\|PtHpa_2Cl_2\|$	3245sh, 3210sbr, 3128m	1579s	318s	260wbr
trans-$\|PtHpa_2Cl_2\|$	3248m, 3219sbr, 3146m	1588s	329s	276wbr
$\|PtHpa_3Cl\|Cl$	3220sh, 3180sbr, 3103m	1585m	343mw	290mbr

The infrared spectra (Table 1.) in the ν(NH) (3300 - 3000 cm^{-1}) and $\delta(NH_2)$ (1650 - 1550 cm^{-1}) regions are strictly related to stoichiometry and geometry, as previously observed for the Hea and Pra analogues (2, 3). As a general trend, the species cis-$|PtL_2Cl_2|$ present a strong broad absorption at ca. 3200 cm^{-1} and a medium intensity one at 3125 cm^{-1} . In the same region the trans analogues show three sharp bands of medium intensity. Due to formation of NH hydrogen bridges with the chlorine ions, the ν(NH) absorptions of the 1:3 and 1:4 complexes are shifted to lower energy, at 3170 cm^{-1} and 3100 cm^{-1} respectively. As expected, the Pt-Cl stretching frequency of the trans complexes is ca. 10 cm^{-1} above the one of the

corresponding cis isomers, apart for the Bua derivatives, having the same ν(Pt-Cl) (320 cm^{-1}) but clearly different ν(NH) and $\delta(NH_2)$ absorptions. All complexes present a broad absorption below 300 cm^{-1} assigned to the stretching of the Pt-N bond.

Table 2. Thermal data

Compound	Decomp.[a]	DTA peaks, °C	Weight loss %[b]
cis-$\|PtPea_2Cl_2\|$	135-330	180m, 262d, 293d	53.6 (55.7;2Pea+2Cl)
trans-$\|PtPea_2Cl_2\|$	190-330	140m, 297d	54.5 (55.7;2Pea+2Cl)
$\|PtPea_3Cl\|Cl$	150-330	185md, 285d	17.1 (16.5;1Pea)
			45.9 (46.5;2Pea+2Cl)
$\|PtPea_4\|Cl_2$	90-330	135m, 173d, 250d,	27.5 (28.3;2Pea)
		267d	39.8 (39.9;2Pea+2Cl)

a) Decomposition interval, °C; m, melting endotherm; d, decomposition endotherm. b) The calculated values are in parentheses.

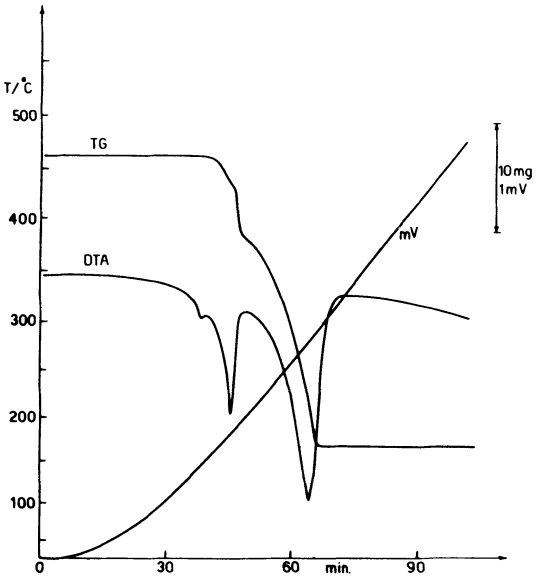

Fig. 1. Thermograms of $|PtPea_3Cl|Cl$ (42.6 mg).

The thermal data of the Pea derivatives are reported as an example of the thermal behaviour of a complex series (Table 2).

The degradation of the 1:2 species immediately follows the melting process, yielding platinum as a final product in a single step.

The decomposition of the higher stoichiometry species takes place in two close steps, the first one due to the release of ligand to give trans-$|PtPea_2Cl_2|$ as an intermediate. In the thermograms of $|PtPea_3Cl|Cl$ (Fig. 1) the first endothermal peak at 185°C is related to melting with subsequent release of one Pea molecule, whereas the stronger endotherm at 285°C concerns trans-$|PtPea_2Cl_2|$ decomposition.

Table 3. ^1H n. m. r. data in $CDCl_3$ (ppm; T, 25°C).

Compound	NH_2 [a]	αCH_2	βCH_2
cis-$|PtPea_2Cl_2|$	4.8	2.76	1.68
trans-$|PtPea_2Cl_2|$	3.5	2.77	1.60
$|PtPea_3Cl|Cl$	6.2-4.6	2.7	1.7
$|PtPea_4|Cl_2$	6.1	2.6	1.7

[a] $J(H-^{195}Pt)$, ca. 60 Hz.

The ^1H n. m. r. spectra are characteristic of the stoichiometry and geometry and allow to detect isomerization processes and isomer mixtures in 1:2 complex samples. As is shown in the Pea series (Table 3), the broad NH_2 resonance is observed at 4.8 ppm in cis-$|PtPea_2Cl_2|$, well downfield with respect to the corresponding signal of trans-$|PtPea_2Cl_2|$ (3.5 ppm). Due to the different environment of ligand molecules in square planar moiety $|PtPea_3Cl|^+$, the 1:3 complex presents two NH_2 signals. The downfield one, at 6.2 ppm, having half the intensity of the stronger one, at 4.6 ppm, is assigned to the Pea molecule trans to chlorine. The NH_2 signals present the satellites due to proton coupling with ^{195}Pt, so excluding release of ligand in the 1:3 and

1:4 complex solution. The stability of the higher stoichiometry complex depends on the halide and follows the order Cl > Br > I (4,5).

The series of the neutral complexes cis-$|PtL_2Cl_2|$ and of the parent trans isomers were tested on tumoral KB cell line culture (L = Pra, Bua, Pea, Hea, Hpa). Moreover cytotoxicity data are reported for some ionic complexes of the type $|PtL_3Cl|Cl$ and $|PtL_4|Cl_2$.

The results are shown in Fig. 2. The cytostatic activity of cis-$|PtL_2Cl_2|$ varies in the order Pra > Bua > Pea $\simeq$ Hea $\simeq$ Hpa. In particular the ID_{50} value for cis-$|PtPra_2Cl_2|$ is two times the value observed for cisplatin in the same conditions.

The above data are in accordance with those observed in experiments on ADJ / PC6 A and L1210 tumours " in vivo " by T. A. Connors et al. (7,8,9).

As expected, the trans isomers are generally less active than the corresponding cis species when tested on KB cells. Among them a significant activity has been observed for trans-$|PtBua_2Cl_2|$ ($ID_{50} \simeq 2.. 10^{-6}$ mol l^{-1}) very close to that of the cis analogue ($\simeq 10^{-6}$ mol l^{-1}).

Cytostatic activity was observed for the charged complexes $|PtPea_3Cl|Cl$ and $|PtHea_3Cl|Cl$. Surprisingly, the $|PtPea_4|Cl_2$ value is very low and close to that of cisplatin.

The ^1H n. m. r. data in $CDCl_3$, indicate the compounds as stable in organic medium. Charged compounds having no labile groups such as $|Pt(NH_3)_4|Cl_2$, $|Pt(NH_3)_2en|Cl_2$ or $|Pten_2|Cl_2$ were found inactive when tested " in vivo ". Moreover $|Pt(NH_3)_3Cl|Cl$ showed no antitumour effect (1). Therefore it will be of interest to extend the study of the above compounds, in attempt to make clear their structure-activity relationship. Attention should be given to the effect of water and chloride ions on the complexes, since the cytostatic activity is measured in an aqueous media containing an appreciable chloride ion concentration.

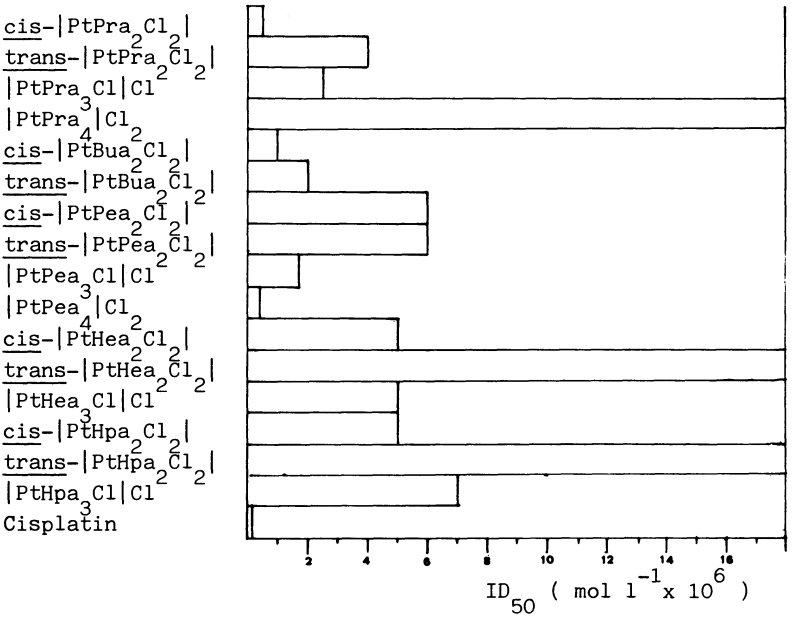

Fig. 2. Cytostatic activity against KB cell growth. The results are expressed as drug concentration for which the cells show a 50 % growth inhibition (ID_{50}). The upper limit criterium for significative cytostatic activity was 10^{-5} mol l^{-1}.

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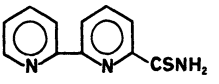
ANTITUMOR ACTIVITY OF METAL(II) CHELATES OF 2,2'-BIPYRIDYL-6-CARBOTHIOAMIDE: *IN VITRO* STUDY

P. Franchetti, E. Nasini, S. Vittori, E. Lepri, G. Nocentini and A. Barzi

INTRODUCTION

Our previous studies directed to the investigation of compounds containing the N*-N*-S* tridentate ligand system as antitumor agents indicated the antileukemic activity of 2,2'-bipyridyl-6-carbothioamide (1) (bpyta).

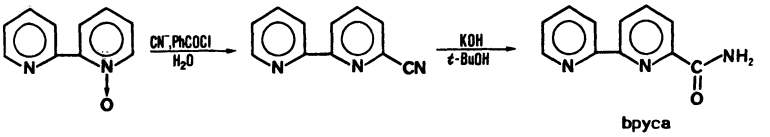


bpyta

The cytotoxic activity of this compound might be correlated to its ability to inhibit ribonucleoside diphosphate reductase, an obligatory enzyme in the pathway of DNA synthesis. On the other hand, several complexes of transition metals with different N*-N*-S* ligand system are endowed with antitumor activity (2). Because of these interesting findings, we prepared complexes of Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) with bpyta.

Furthermore, in order to investigate the role of the sulphur atom of the ligand we have also synthesized the 2,2'-bipyridyl-6-carboxamide (bpyca) (Scheme 1) and its Cu(II) and Fe(II) complexes.

SCHEME I



bpyca

PREPARATION OF COMPLEXES

The monoligand complexes of bpyta and bpyca were prepared by reacting a solution of the ligand in ethanol with equimolecular ethanolic solutions of the metal(II) salts. The bis-ligand complexes of bpyta with Fe(II), Co(II) and Ni(II) were obtained using an excess of ligand (molar ratio 2:1).

BIOLOGICAL ACTIVITY

Aim of the present study was to investigate the in vitro antiproliferative activity of these complexes with respect to bpyta and bpyca. For this purpose a short term assay based on the incorporation of ^{125}Iododeoxyuridine into DNA of P388 murine lymphocytic leukemia and HL-60 human acute promyelocytic leukemia cell line was used.

MATERIALS AND METHODS

DRUG EFFECTS ON INCORPORATION OF ^{125}IODODEOXYURIDINE INTO DNA OF LEUKEMIC CELLS.

2,2'-bipyridyl-6-carbothioamide, 2,2'-bipyridyl-6-carboxamide and their metal(II) complexes were tested on P388 murine lymphocytic leukemia, maintained by weekly i.p. injection of 10^5 or 10^6 cells in histocompatible (CD2F1) mice and on HL-60 human acute promyelocytic leukemia cell line, cultured in vitro. Tumor cell suspensions were incubated with ligands (dissolved in DMSO) or complexes (dissolved in H_2O) for 1 hr. Thereafter, the cells were washed twice, suspended and placed, in quadruplicate, in flat-bottomed microculture wells for 48 hr. Antiproliferative activity was determined by adding to the cultured cells ^{125}IUdR together with 5-fluoro-2'-deoxyuridine, 48hr after plating for an additional 18 hr. The results are expressed as % inhibition of radioisotope incorporation in the treated cultures with respect to untreated controls.

DRUG EFFECTS ON VIABLE CELL NUMBER

Differences in viable cell numbers between control and treated cultures were determined, as for the ^{125}IUdR incorporation assay 48hr after plating, by Tripan blue dye exclusion test performed in quadruplicate. The drug-induced inhibition of cell survival, expressed as % reduction of viable cells in the treated cultures with respect to untreated controls, was calculated as follows:

$\%R = \frac{a - b}{a} \times 100$, where "a" is the mean of viable cell numbers in controls and "b" is the mean of viable cells in treated cultures.

RESULTS AND CONCLUSION

As preliminary screening, the antitumor activity against P388 and HL-60 leukemic cells of bpyta and its metal(II) complexes was investigated in vitro using a biochemical assay which measured radiolabelled precursor incorporation into DNA. The effect of these compounds on survival of tumor cells was also tested by directly counting tumor cells in liquid culture. Results of at least 3 experiments carried out in quadruplicate are shown in Tables I and II. The IC_{50} values, obtained from dose-effect curves, indicate that bpyta is active in inhibiting DNA synthesis in vitro both on P388 and HL-60 cells (Table I). The Fe(II) complexes with bpyta show antiproliferative activity in vitro against P388 cells, generally like the metal-free ligand and the Co(II) and Zn(II) complexes, whereas Ni(II) and Cd(II) complexes do not exhibit any activity at the concentrations tested. In agreement with literature data concerning structurally similar ligands (3, 4) the copper complex with bpyta was the most active compound. This pronounced effect cannot be explained solely on the basis of enhanced uptake of the metal complex and it cannot be the metal, rather than the drug component of the complex, which exerts ultimate toxicity, as $CuCl_2$ when tested at equimolar concentrations was about seven-fold less active than the copper complex with bpyta. Bpyta may, however, reach previously inaccessible sites when chelated to Cu^{++} (5).

The comparison between inhibition of cell multiplication and reduction of viable cell number of bpyta and its copper and iron complexes performed on P388 cells is shown in Table II. The results obtained using equimolar concentrations of the compounds confirmed the greatest activity of the copper complex both in terms of DNA damage and cell survival. In addition, in aqueous solution and in the presence of Cu ions, bpyta exhibits antiproliferative activity comparable to the copper complex with bpyta.

In order to investigate the role of the sulphur atom, bpyca was synthesized and tested in vitro for antitumor activity against P388. As shown in Table I substitution of the sulphur atom in bpyta with the

TABLE I Inhibition of ^{125}IUdR incorporation into DNA of P388 or HL-60 leukemic cells by 2,2'-bipyridyl-6-carbothioamide, 2,2'-bipyridyl-6-carboxamide and their metal (II) complexes.

Compound	* IC_{50} μM P388	* IC_{50} μM HL-60
bpyta	315.87	588.62
$[Fe(bpyta)(H_2O)]SO_4$	453.00	inactive
$[Fe(bpyta)_2(H_2O)_6]SO_4$	273.90	inactive
$[Co(bpyta)(H_2O)_2]Cl_2$	339.00	◊ NT
$[Co(bpyta)_2(H_2O)_3]Cl_2$	282.00	NT
$[Ni(bpyta)(H_2O)]Cl_2$	inactive	NT
$[Ni(bpyta)_2(H_2O)_4]Cl_2$	inactive	NT
$[Cu(bpyta)]Cl_2$	25.00	55.98
$[Zn(bpyta)]Br_2$	235.00	250.62
$[Cd(bpyta)]I_2$	inactive	NT
bpyca	inactive	inactive
$[Cu(bpyca)(H_2O)]Cl_2$	129.56	373.71
$[Fe(bpyca)(H_2O)]SO_4$	240.63	inactive
$FeSO_4$	576.64	NT
$CuCl_2$	212.36	NT

*; Micromolar concentration required to inhibit ^{125}IUdR incorporation by 50% of untreated controls. Values obtained from dose-effect curves. At least three concentrations were used for each compound studied.

◊; No tested.

TABLE II - Comparative evaluation of antiproliferative activity of bpyta and its iron and copper complexes, carried out at an equimolar concentration (71 μM) of the compounds. Results obtained using ^{125}IUdR uptake and Tripan blue dye exclusion tests.

Compound	% inhibition of ^{125}IUdR uptake	% reduction of viable cell number
bpyta	0	0
$[Fe(bpyta)(H_2O)]SO_4$	17	42
$[Cu(bpyta)]Cl_2$	100	97
bpyta + $CuCl_2$	100	94
$CuCl_2$	2	44

oxygen one gave an inactive compound. However, the inhibitory effect on DNA synthesis exerted on P388 and HL-60 cells by the copper complex of bpyca might indicate that tridentate ligand systems other than the N*-N*-S* ones could have antitumor properties.

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TERNARY COMPLEXES OF Pt^{+2} AND Pd^{+2} WITH INO, GUO AND AMINO ACIDS

A. Garoufis, S. Kasselouri, J.P. Laussac and N. Hadjiliadis

INTRODUCTION

Nucleic acids and proteins interact through specific amico acid side chains and nucleic acid bases in biological systems and this may be done with the aid of a metal ion, forming a ternary compex (1,2). The compounds **cis**-$Pt(ino)_2Cl_2$ and **cis**-$Pd(guo)_2Cl_2$ were allowed to react with various amino acids, in order to detect such nucleoside-amino acid interactions, as a first attempt. This may substantiate earlier suggestions of **cis**-DDP produced DNA-protein crosslinks (3), exhibiting its anticancer properties.

MATERIALS AND METHODS

(a) **Materials**: Glycine, L-Alanine, L-Valine, L-Isoleucine, L-Proline, L-Phenylalanine, Inosine and Guanosine were purchased from Aldrich Chemical Company and Fluka A.G.Potassium tetrachloroplatinate and Palladium chloride were from Lyon allemand (Paris, France).

(b) **Methods**: (i) General method for the preparation of the complexes **cis**-$[M(nucl)_2(am\text{-}ac)]Cl$.

1 mmol of each of the compounds **cis**-$Pt(ino)_2Cl_2$ and **cis**-$Pd(guo)_2Cl_2$ and 1.2 mmols of the corresponding am-acNa were mixed in the solid state and 200ml of methanol added. The suspensions were stirred at 30°C for 24 hrs. They were then filtered through filter papers from

the unreacted materials and the yellow filtrates evaporated to dryness. They were disolved in a small volume of DMF and filtered from the insoluble NaCl and other insoluble materials. They were then precipitated out from the DMF solutions, by adding excess of acetone: ether (1:1) and filtered and dried on air. They were then recrystallized in mixtures of ethanol: water=8:2 and dried first at room temperature and then at 110°C under vacuum. The yields varied from 50-75%.

(ii) General method for the preparation of the complexes **cis-** $Pt(nucl)_2(am\text{-}ac)Cl$ Cl.

1 mmol of each of the complexes **cis-**$[Pt(ino)_2(am\text{-}ac)]Cl$ and **cis-**$[Pd(guo)_2(am\text{-}ac)]Cl$ were disolved in the equivalent amount of aqueous solutions of HCl (∿5ml) and the mixtures were allowed to react for about 16 hrs for the platinum series but only 10-15 minutes for the palladium analogs, at room temperature. After filtering the solutions, the complexes were precipitated with isopropanol and Ether (1:1), filtered and dried first at room temperature and then at 110°C under vacuum. The yields were quantitative.

RESULTS

The 24 new complexes isolated with the above general methods and corresponding to the formulae **cis-**$[Pt(ino)_2(am\text{-}ac)]Cl$, **cis-**$[Pd(guo)_2(am\text{-}ac)]$ Cl, **cis-**$[Pt(ino)_2(am\text{-}acH)Cl]Cl$ and **cis-**$[Pd(guo)_2(am\text{-}acH)Cl]Cl$ give satisfactory elemental analysis and are 1:1 electolytes in aqueous solutions.

Both nucleosides are coordinated with the metals through their N_7 atom and they retain this coordination in their ternary complexes with amino acids. The amino acids are N,O coordinated as their ir and ^{1}Hnmr spectra show (4,5).

The small chemical shifts of the protons of the coordinated amino acids in the ^{1}Hnmr spectra of the

complexes, were explained as due to ligand-ligand hydrophobic interactions in the Pt(II) series of complexes (5). The same behavior is also observed in the Pd(II) analogs.

In the first series of complexes with Pd(II) two main resonances are observed, due to the H_8 proton of guo, in D_2O solutions of almost the same intensity. For example for **cis**-$[Pd(guo)_2(Val)]Cl$ at 8.43 and 8.13 ppm. The spectrum of the same compound in DMSO-d_6 solutions show the two H_8 resonances at 8.60 and 8.04 ppm correspondingly, but the resonance at lower frequency is at least three times larger than the other. This shows the existence of two isomers in equilibrium, sensitive to the solvent. Fig.1.

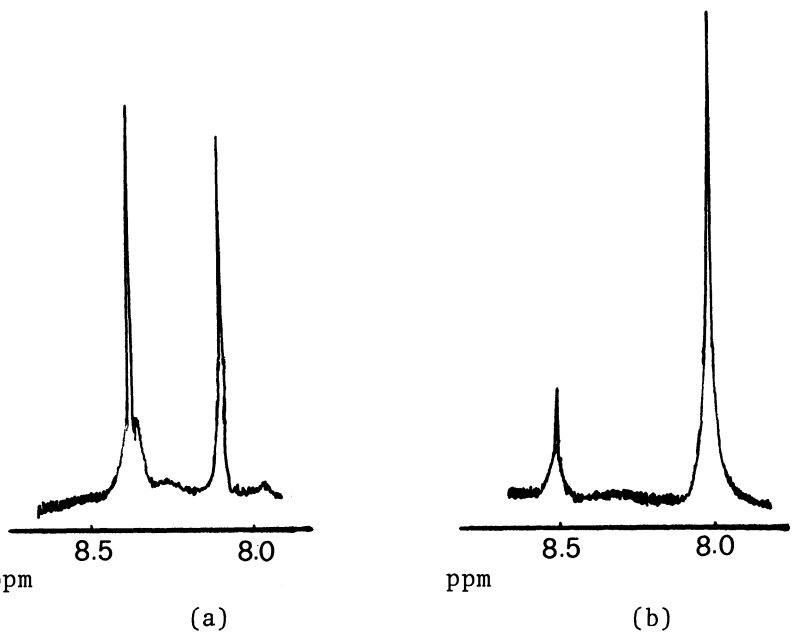

Fig.1. The ^{1}Hnmr spectrum of **cis**-$[Pd(guo)_2(Val)]Cl$ in (a) D_2O and (b) DMSO-d_6.

The possible type of isomers in solution are not known with certainty at present, but they are postulated

to be due to the tendency of Pd(II), to react with the exocyclic O_6 of guo (6) thus compeding with the carboxylate oxygen atom. This tendency is lower in DMSO-d_6 but it is not completely eliminated due to the presence of some water in the solvent.

In the second series of complexes with Pd(II) more resonances are found in the ^{1}Hnmr spectrum for the H_8 proton of guo and these were assigned to different rotational isomers corresponding to head-to-head and head-to-tail arrangements of the guo ligands, with a $\Delta G^{\neq}$ value larger than 76 KJ/mol (4).

The complexation of Pt(II) with inosine was found to increase the C_3' - **endo-anti** conformation of the sugar moiety of the nucleoside from 46% of the free ligand to 59% in the complex **cis**-Pt(ino)$_2$Cl$_2$ (5) and to 50-55% in both series of complexes. Similar behavior is observed in the Pd(II) analogs giving values around 50% for the ternary complexes and 38% only for the free ligand (7). This is true for both isomers of Pd(II) complexes.

The gg percentage of the sugar part of inosine ranging from 71-80% for the Pt(II) complexes (5) is found to range from 70-77% for the one of the isomers of the Pd(II) series, while in the second isomer, range from 60-68%. See Table 1.

Table 1. The various sugar conformations of the compounds.

Compounds	gg%	tg,gt%	%^{3}E	K_{eq}
cis- [(Guo)$_2$Pd(Val)]Cl	69	31	52	1,08
	77	23	50	1,00
cis- [(Guo)$_2$Pd(Ileu)]Cl	67	33	50	1,00
	75	25	49	0,96
Guanosine	69	31	38	0,61
cis- [(Ino)$_2$Pt(Val)]Cl	77	23	50	1,00
cis- [(Ino)$_2$Pt(Ileu)]Cl	73	27	50	1,00
Inosine	74	26	46	0,85

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SYNTHESIS AND *IN VITRO* ANTINEOPLASTIC ACTIVITY OF DIORGANOSTANNYLENE DERIVATIVES OF 2,6-PYRIDINE DICARBOXYLIC ACID

M. Gielen, E. Joosen, T. Mancilla, K. Jurkschat, R. Willem, C. Roobol and J. Bernheim

INTRODUCTION

The widespread success of platinum compounds in the clinical treatment of testicular and ovarian cancers for instance, has stimulated research in the area of metal-based anti-tumour drugs and spurred the search for organometallic compounds with improved therapeutic properties.

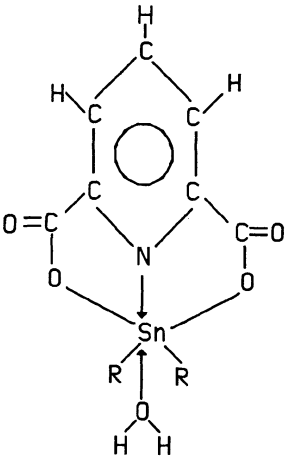
In general, the complexed or uncomplexed organotin compounds of the type R_2SnX_2 tested until now against P388 lymphocytic leukemia in mice only showed marginal activity (1), even if almost 50% of the tested diorganotin dihalides do exhibit some activity (2).

We have booked some promising *in vitro* activities for the di-n-butylstannylene derivatives of pyridoxine, cortexolone and erythromycine, three more or less complex diols (3).

SYNTHESIS AND CHARACTERIZATION OF $C_5H_3N(COO)_2SnR_2.HOH$

Therefore, we have prepared some diorganostannylene derivatives of 2,6- pyridine dicarboxylic acid by the simple condensation $C_5H_3N(COOH)_2 + OSnR_2 \rightarrow C_5H_3N(COO)_2SnR_2.HOH$ (R = CH_3, compound **1**; R =

$CH_2CH_2CH_2CH_3$, compound **2**; R = $C(CH_3)_3$, compound **3**; R = C_6H_5, compound **4**)



Their structure and purity has been determined by IR, ^1H and ^{13}C NMR, and by mass and Mössbauer spectrometry (5). They are seven-coordinate in the solid state, as shown by X-Ray diffraction (5).

IN VITRO SCREENING

The *in vitro* cytotoxic effects of compounds **1** to **4** (5) have been compared with those of "classical" antineoplastic agents : compound **2** shows against P388 an in vitro activity that is fourteen times that of cis-platin, a clinically frequently used metal-based antitumour agent; it has an activity comparable to that of methotrexate. Against P815, compound **2** is more than 30 times as active as cis-platin and shows an activity comparable to that of vincristin. Against L1210, it is about six times more active as cis-platin and exhibits an activity comparable to that of cytosine-arabinoside. Compound **4** is another rather active compound within the series here: it appears to be about five times as active as cis-platin against L1210 and P388, and exhibits an activity comparable to that of cytosine-arabinoside against these leukemiae,

and it is more than twenty times as active as cis-platin against P815 leukemia and B16 melanoma, showing in those cases an activity that is comparable to that of vindesin; it is two times as active as cis-platin against Murine Lewis Lung carcinoma and shows here an activity comparable to that of daunorubicin. Compound **3** appears to be about two times as active as cis-platin against L1210 and P388, about ten times as active as cis-platin against P815 and B16 melanoma, and about as active as cis-platin against Lewis'carcinoma. It exhibits an activity comparable to that of mitomycin-C against L1210 leukemia, B16 melanoma and Lewis Lung carcinoma. Only compound **1** appears to be inactive. These data encourage the undertaking of further studies of the potential antitumour activity of tin-based organometallic compounds.

Table 1. In vitro screening of some diorganostannylene derivatives of some 2,6-pyridine dicarboxylic acid and of cis-Platin

	Murine L1210 leukemia	Murine P815 leukemia	Murine P388 leukemia
Compound tested	IC50, μM	IC50, μM	IC50, μM
Compound **2**	0.10	0.051	0.017
Compound **4**	0.11	0.071	0.05
Compound **3**	0.29	0.18	0.14
cis-Platin	0.65	1.5	0.25
Compound **1**	17	11	4.5
	Murine B16 melanoma	Murine Lewis Lung carcinoma	
Compound tested	IC50, μM	IC50, μM	
Compound **4**	0.062	0.4	
Compound **3**	0.12	0.81	
cis-Platin	1.7	0.88	
Compound **1**	4.8	56	

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TWO NEW WATER SOLUBLE PLATINUM COMPLEXES - CL 286, 558 AND CL 287, 110

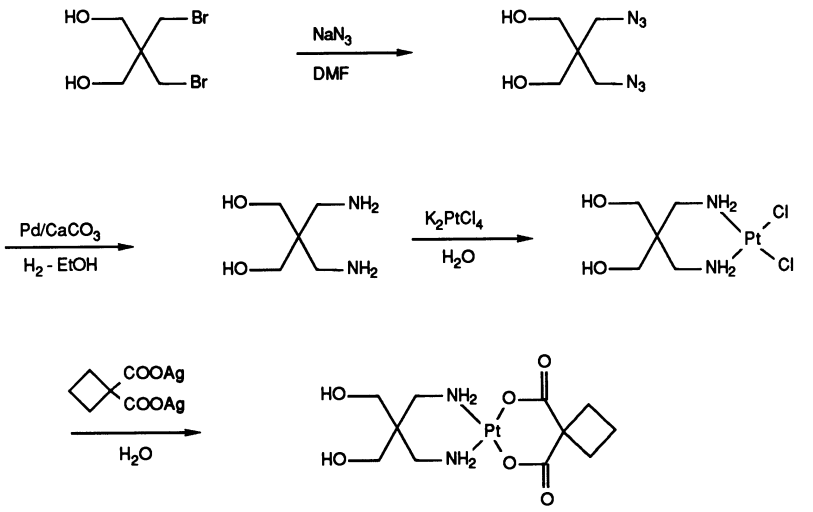
J.J. Hlavka, P. Bitha, S.G. Carvajal, R.G. Child, F.E. Durr, S.A. Lang, Jr., Y-I Lin, H.L. Lindsay, J.P. Thomas and R.E. Wallace

INTRODUCTION

Several second generation platinum complexes having diaminocyclohexane or 1,1-bis(aminomethyl)cyclohexane as the stable amine ligand have entered clinical trials. These complexes are (1,2-diaminocyclohexane)malonatoplatinum, aqua[1,1-bis(aminomethyl)cyclohexane]sulfatoplatinum (Spiroplatin), (4-carboxyphthalato)-(1,2-diaminocyclohexane)platinum, and (1,2-diaminocyclohexane)-(isocitrato)platinum (PHIC). Although these compounds exhibited excellent antitumor activity and lack of cross-resistance with DDP, none of them appear to have any future for further clinical evaluation. The major difficulties encountered to date are insufficient water solubility, excessive host toxicity, inadequate purity and lack of acceptable formulation. In order to improve on these drawbacks, we have used a malonate derivative for the carboxylate ligand and incorporated oxygen into the amine ligand and/or the carboxylate ligand, and a few hundred platinum complexes have been synthesized.

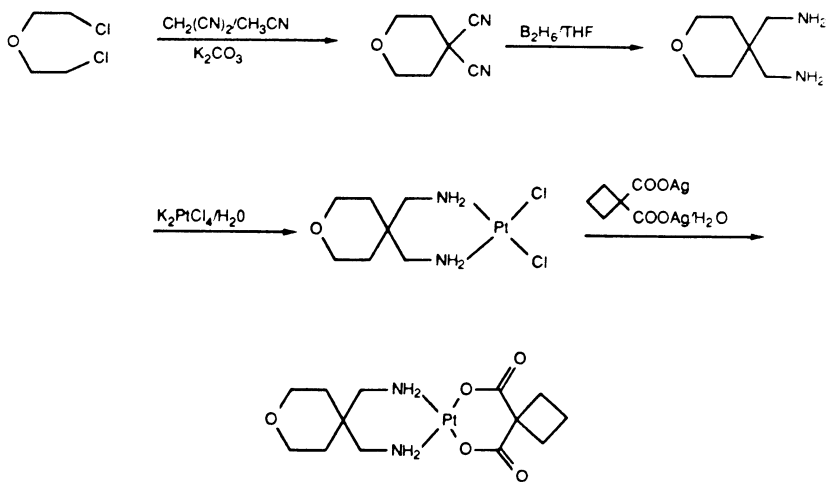
This poster describes the synthesis and biological properties of the two most interesting members of the series, CL 287,110 and CL 286,558 in which the labile ligand is 1,1-cyclobutanedicarboxylate and the stable amine ligand is either 4,4-bis(aminomethyl)tetrahydro-4H-pyran or 2,2-bis(aminomethyl)-1,3-propanediol.

Synthesis of CL 286,558



CL 286,558

Synthesis of CL 287,110



CL 287,110

Platinum Compounds: Effective Dose Range

Drug	Effective Dose Range[1]		Therapeutic Ratio[2]
	Optimum	Minimum	
CL 286,558	6	0.2	32
CL 287,110	100	3	32
Platinol™	2	0.5	4
JM-8	50	≤3	≥16

[1] Against P338 leukemia in mice treated IP on days 1, 5 and 9 after IP tumor implant.
[2] Optimum effective dose divided by minimum effective dose.

Platinum Drugs: Relative Activity Against Human Tumors in Mice

Drug[2]	% Tumor Weight Reduction at Optimal Dose[1]	
	Breast (MX-1)	Ovarian (H207)
CL 286,558	71	200
CL 287,110	92	200
Platinol™	186	186
JM-8	90	200

[1] Tumor weight reduction ≥58% is significant.
[2] IP on days 8, 12 and 16 after SC tumor implant

Platinum Compounds: Relative Water Solubility

Drug	Max. Tol. Dose[1]		Water Solubility	
	Mg/kg	Mg/Mouse	Mg/ml	Doses/ml[2]
CL 286,558	6	0.12	4.9	40
CL 287,110	100	2	10	5
Platinol™	2	0.04	1	25
JM-8	50	1	≥10	≥10

[1] Given IP on days 1, 5 and 9 after IP P388 leukemia implant.
[2] The number of maximum tolerated doses soluble in one ml of water (mg/mouse dose divided into mg/ml soluble in one ml of water).

Summary of Some Important Characteristics

	CL 287,110	CL 286,558	Platinol	JM-8
Rodent Nephrotoxicity[1]	non-toxic	non-toxic	toxic	non-toxic
Therapeutic Dose Ratio[2]	32	32	4	$\geq$ 16
Water Solubility[3]	4.9	40	25	10
Stability in Water	stable[4]	stable[4]	unstable	stable[4]

[1] Significant elevation of BUN; $\geq$ 20% over normal is rated toxic.
[2] Optimal therapeutic dose/minimal effective dose based on the P388 screen.
[3] The number of maximum tolerated doses soluble in 1 ml of water.
[4] Less than 2% degradation after 24 hours.

Platinum Compounds: Relative Activity Against Murine Tumors[1]

	% ILS1 (% survivors) at Optimal Dose in Mice					
		Leukemias			Solid Tumors	
Drug	P388	L1210	L1210 CPR[2]	B-16	Colon 26	M5076
CL 286,558	91 (6)	36	19	135 (25)	55	>140 (90)
CL 287,110	145 (50)	142 (33)	>275 (67)	48	121	>114 (80)
Platinol™	140 (17)	96 (4)	8	64	53 (6)	73 (37)
JM-8	144 (6)	35	13	70 (13)	85 (17)	>115 (80)

[1] An ILS (increase in life span) > 25% is considered significant; % survivors at 30 days for leukemias; at 60 days for solid tumors. Tumor implantations and drug treatments were by the intraperitoneal route.
[2] CPR = cisplatin resistant.

CONCLUSION

These two platinum complexes, CL 287,110 and CL 286,558, exhibit a high degree of antitumor activity, low renal toxicity and lack of cross-resistance with DDP. Because of these favorable biological attributes, as well as their ease of synthesis, stability in solution and excellent solubility, they are currently under consideration for further development.

COMPLEXES OF WATER SOLUBLE POLYMERS AND ORGANOPLATINUM COMPOUNDS AS POTENTIAL TIME-RELEASE ANTITUMOR FORMULATIONS

B.A. Howell, E.W. Walles, R. Rashidianfar, J.R. Glass, B.J. Hutchinson and D.A. Johnson

INTRODUCTION

The serendipitous discovery of the biological activity of cis-dichlorodiammineplatinum(II) ["Cisplatin"] by Rosenberg in 1965 spawned an intensive investigation of the chemical and biological properties of this compound and its derivatives (1-6). Cisplatin and derivatives represent a class of very effective, broad spectrum antitumor agents (6). These compounds are readily available by synthesis, are chemically well-behaved and are effective against a broad spectrum of tumors. Yet the use of these drugs has generally been limited to the treatment of advanced ovarian and testicular cancers. The severe side effects which accompany the administration of these compounds have prevented the wide-spread exploitation of the potential which these drugs seem to offer (7-14). Principal, among the side effects induced by the organoplatinum drugs is 1) severe kidney damage (7) and 2) extreme nausea (11, 14). To permit a more general use of these compounds for the treatment of cancer, the side effects must be substantially reduced in intensity or removed. While forced-hydration therapy has met with some success, it is sufficiently cumbersome and ineffective so as to preclude the more general administration of organoplatinum compounds for the treatment of most forms of cancer (14-17). Obviously, then, the development of a system which retains the effectiveness of the organoplatinum drug while suppressing or eliminating the principal side effects would be a boon to the field of chemotherapy.

An initial approach for the improvement of the properties of cisplatin was the preparation of analogs in which the amine ligands were varied through a wide range of structure. In addition, organoplatinum compounds which incorporate non-amine inert ligands have been prepared (few of these

compounds display antitumor activity). Literally, hundreds of organoplatinum compounds have been prepared, characterized and evaluated for antitumor activity (18).

As one consequence of this screening, it was found that the replacement of the ammonia ligands of cisplatin with 1,2-diaminocyclohexane appears to reduce the toxicity while increasing the antitumor activity (19). These effects are also observed for other compounds containing the 1,2-diaminocyclohexane ligand along with highly labile leaving groups. One such early compound was 4-carboxyphthalato(1,2-diaminocyclohexane)platinum(II). By virtue of the free carboxyl group, this compound is readily soluble in physiologically compatible 1% aqueous sodium bicarbonate solution. In addition, the toxicity displayed by this compound is somewhat lower than that of dichloro(1,2-diaminocyclohexane)platinum(II) or cisplatin (19-22). Using a similar approach, the (1,2-diaminocyclohexane)platinum moiety can be made water soluble by coordination with N-phosphonacetyl-L-aspartate (a ligand which alone displays some antitumor activity). The resulting compound, N-phosphonacetyl-L-aspartato-(1,2-diaminocyclohexane)platinum(II), is highly water soluble due to the presence of a terminal phosphate group. This compound displays activity against a wide variety of tumors (20, 24). A water soluble antitumor agent can also be generated by coordination of (1,2-diaminocyclohexane)platinum with isocitrate (25).

Another water soluble analog of cisplatin which has shown promise in phase I clinical trials is bis-pyruvato(1,2-diaminocyclohexane)platinum(II) (26). Although the immuno suppressive properties of the compound is similar to that of other organoplatinum compounds (27), its antitumor effect for leukemia L1210 is greater than that of cisplatin (25 fold improvement in therapeutic index) (28).

Two compounds which seem to hold most promise for cancer chemotherapy are 1,1-cyclobutanedicarboxylato(diammine)platinum(II) (carboplatin) and cis-dichloro-trans-dihydroxo-cis-bis(isopropylamine)platinum(IV) (iproplatin). Carboplatin is much less emetic than cisplatin and displays virtually no renal toxicity (29-37). Thus it displays much potential as a second generation organoplatinum drug. Thrombocytopenia is the dose limiting side effect for this compound (30). Iproplatin displays a wide spectrum of antitumor activity and lack of renal toxicity (37-41). The dose limiting toxicity is myleosuppression, particularly thrombocytopenia (42).

A new platinum containing antitumor compound has recently been developed which has a structure very different from that of cisplatin and analogs (43, 44). This compound, ascorbato(1,2-diaminocyclohexane)platinum(II), has several characteristics which distinguish it from the more traditional platinum compounds. These include:

1) ascorbate acting as a bidentate ligand

2) the presence of a platinum-carbon bond; and

3) activity by both cis and trans isomers.

This compound has displayed antitumor activity equal to or greater than that of cisplatin in a variety of tumor screens. This compound derived from vitamin C is the first carbon-bound analog of cisplatin to display good antitumor activity in vitro (43). The mode of action of this compound may be somewhat different from that of traditional platinum compounds since facile cleavage of the platinum carbon bond under physiological conditions seems unlikely.

Although much effort has been directed to the synthesis of new cisplatin analogs with reduced toxicity, this approach has not yielded any dramatic progress toward this goal. Because of the great desirability of establishing drug formulations for which the side effects associated with the administration of organoplatinum compounds are substantially diminished or removed, several other more novel approaches have been explored. In general these have involved the attachment of an active platinum compound to a suitable carrier vehicle. The potential advantages of the use of such combinations include:

1) restricted biological movement;

2) controlled release;

3) increased probability of critical attachment (depending upon the molecular weight of the carrier) and decreased toxicity; and

4) delivery of increased amounts of the drug.

Carrier species have included known steriod drugs modified to contain a thioacetamide group (45), macrocyclic nitrogen compounds (azacrowns) (46), amino acids (47), natural proteins (serum albumin) (48), polyethylene imine (49), polyglutamic acid (50), polyhydrazines (51), polyvinylamine-covinylformate (52), polyphosphazenes (53-57), and organometallic polymers in which the cis-dichloroplatinum moiety is included in the repeat unit (58-64). Two of these, attachment of the cis-dichloroplatinum moiety to a polyphosphazene and the synthesis of organoplatinum polymers, have received considerably more attention than the remainder. While many of these formulations display

activity toward a variety of tumor cell lines, none would seem likely to overcome the problems associated with the use of cisplatin (64).

A novel and potentially more useful approach to the preparation of polymer-supported, time-release organoplatinum antitumor formulations would be to prepare platinum compounds which, while retaining the essential features necessary for antitumor activity, could be noncovalently bound to a water-soluble polymer. The platinum compound-polymer complex could then be administered as the chemotherapeutic agent. Hydroloysis of the complex in vivo might slowly release the platinum compound, i.e., the bound polymer would serve as a time-release agent, such that the free platinum compound would never be present at concentrations sufficient to induce intolerable side effects. We have initiated a program designed to test the potential offered by this approach (69).

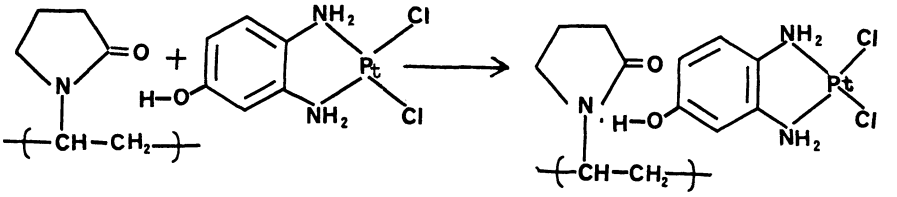
RESULTS AND DISCUSSION

The first requirement for the preparation of complexes of platinum compounds and a water-soluble polymer was to identify a suitable polymeric substrate. While several polymers are capable of forming molecular complexes with appropriately-substituted organoplatinum compounds, the polymer of choice for use in a controlled-release formulation would seem to be poly(N-vinvylpyrrolidone) (PVP). Poly(N-vinylpyrrolidone)

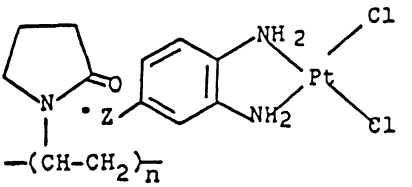
1) is readily available in medical grade and in several molecular weight ranges
2) is water soluble
3) has long been used as a blood extender with no known toxicity
4) has FDA approval for use in drug and food applications
5) is widely used as a clarification agent in the beverage industry
6) forms stable molecular complexes with aromatic compounds bearing polar functional groups (65-68).

In the first instance, 4-substituted o-phenylenediamines were selected as the ligands to be used for the preparation of suitable platinum compounds. A polar substituent at the 4-position provides a "handle" for the complexing interaction with the polymer while the 1,2-amino functions provide the necessary cis amine structure for the formation of platinum compounds analogous to cisplatin. These ligands could be converted to the corresponding platinum compounds by treatment with potassium tetrachloroplatinate(II) in aqueous solution. Treatment of solutions of the platinum compounds in

dimethylformamide with aqueous solutions of PVP afforded the corresponding polymer complexes. This is illustrated below for the interaction of cis-dichloro-(4-hydroxy-o-phenylendiamine)platinum(II) with poly(N-vinylpyrrolidone).



Complexation occurs via a dipolar interaction between the polar aromatic hydroxyl grouping of the platinum compound and the amide functionality of the polymer. This method is rather general and polymer complexes of organoplatinum compounds derived from a variety of 4-substituted o-phenylene-diamine ligands have been prepared (70). In general, these PVP complexes may be formulated as:



Z = -OH, -COOH, $-SO_3H$, or other polar functional group

n = 90 - 350

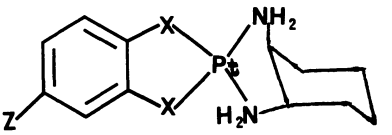
While solutions of these complexes display activity in bacterial screens, organoplatinum compounds derived from aromatic amines as inert ligands generally display somewhat lower antitumor activity than do those generated from aliphatic amines. Therefore, it was desirable to prepare polymer complexes of platinum compounds containing aliphatic amines as inert ligands. In particular, as noted above, the most useful compounds might be those containing 1,2-diaminocyclohexane as the amine ligand. However, this would then require that some aromatic leaving group of lability comparable to that

of chloride be utilized to:

1) maintain the proper hydrolytic reactivity for function as an antitumor agent; and

2) provide a polar aromatic group for complexation with the polymer

Compounds of the following type where Z is a polar group and X is an atom or group of atoms capable of coordinating platinum with approximately the same effectiveness as chloride would be required. Two sets of ligands which might be utilized for the preparation of such compounds may be derived



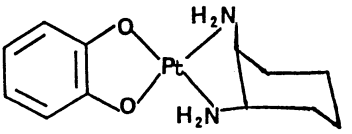
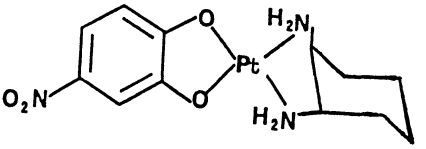
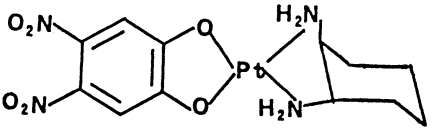
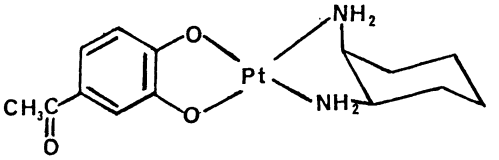
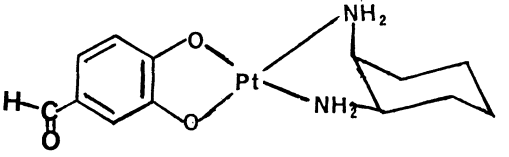
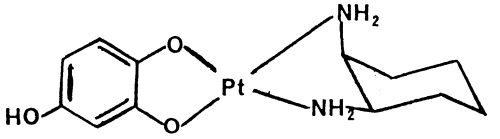
from catechol and phthallic acid. That is, compounds containing the (1,2-diaminocyclohexane)platinum moiety coordinated to 4-substitutedcatecholato ligands or 4-substitutedphthallato ligands to function as:

1) polar handles for attachment to the carrier polymer; and

2) labile leaving groups.

Because of the greater ease of preparation we chose to first examine the catecholato series of compounds. In general, ligands were prepared by the action of an appropriate electrophilic reagent on the 2-butanone ketal of commercially available catechol followed by suitable modification of the functionality introduced and hydrolysis of the ketal. In cases in which the substituent was either hydrogen or an electron-withdrawing group, treatment of cis-dinitrato(1,2-diaminocyclohexane)platinum(II) with the catechol ligand afforded the corresponding catecholato(1,2-diaminocyclohexane)platinum(II) compounds. These compounds were obtained by precipitation from aqueous solution and characterized spectroscopically. The platinum-nitrogen and platinum-oxygen infrared stretching frequencies for some of these compounds are listed in Table 1.

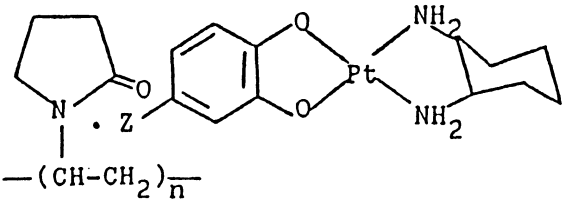
Table 1. Metal-Ligand Absorption Bands in the Infrared Spectra of Catecholato(1,2-diaminocyclohexane)platinum(II) Compounds

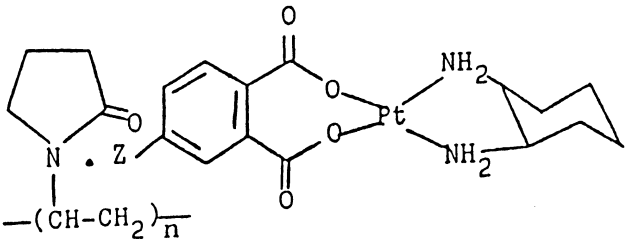
Compound	Absorption (cm^{-1}) Pt-N	Pt-O
	537,482	383,342
	506,421	383,338
	504,439	399,344
	510,440	360
	510,440	340
	510,440	360

The inhibition of DNA crosslinking is one method which may be used to evaluate the potential antitumor activity of organoplatinum compounds. In this procedure, the platinum compound is incubated with DNA under a set of standard conditions. The DNA is then denatured and examined by ultraviolet spectroscopy to establish the extent of interstrand crosslinking, i.e., inhibition of denaturation which has occurred. Two of the compounds generated in this work, catecholato(1,2-diaminocyclohexane)platinum(II) and 4,5-dinitrocatecholato(1,2-diaminocyclohexane)platinum(II), were examined by this technique. Both brought about interstrand crosslinking much more rapidly than did cisplatin used as a control. This suggests that catecholato and 4,5-dinitrocatecholato are considerably better leaving groups than is chloride. In fact, the rate of hydrolysis of the catecholato ligands may be so great as to diminish the effectiveness of organoplatinum compounds derived from these ligands as antitumor compounds.

In general, treatment of cis-dinitrato(1,2-diaminocyclohexane)platinum(II) with a catechol ligand bearing an electron-releasing substituent in the 4-position, with a few notable exceptions, led to the rapid formation of the corresponding quinones and platinum(0) which appeared as a mirror on the walls of the reaction flask.

Because of the limitations associated with the catecholato compounds, both with respect to the variety of structures possible and to biological activity, we have now focused on the preparation of a series of 4-substituted-phthallato ligands, the corresponding (1,2-diaminocyclohexane)platinum(II) compounds and the polymer complexes to be derived from them. In particular, the PVP complexes of 4-hydroxy- and 4-carboxyphthallato(1,2-diaminocyclohexane)platinum(II) seem to hold much promise as suitable time-release formulations. Structures for the catecholato and phthallato polymer complexes are shown below.





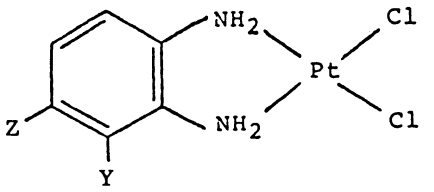
Z = -OH, -COOH, $-SO_3H$, $-SO_2NR_2$, etc.

n = 90 - 350

METHODS AND MATERIALS

cis-Dichloro(substituted o-phenylenediamine)platinum(II) Compounds

The necessary substituted o-phenylenediamine ligands were readily synthesized from commercially available starting materials. Treatment of these ligands with potassium tetracholoroplatinate(II) in aqueous acidic solution at 50°C afforded the corresponding platinum compounds in good yield (71). The platinum compounds, (structures shown below), were characterized by spectroscopic methods and by elemental analysis.



Z	Y
H	H
OH	H
H	OH
COOH	H
OCH_3	H
SO_3H	H

The proton nmr spectra of all of the compounds contain complex absorption patterns due to the overlapping of the signals due to the aromatic protons and the amino protons. The infrared spectrum of each of the compounds contains a strong band at 3200 cm^{-1} attributable to the presence of the amino group in the compound. The spectrum of cis-dichloro(3,4-diaminobenzoic acid)platinum(II) contains a carbonyl absorption on 1730 cm^{-1} (KBr). None of the platinum compounds melt, even at 400°C, except for cis-dichloro(3,4-diaminobenzoic acid), which decomposes at 380°C.

Complexes of Organoplatinum Compounds with PVP

In general, treatment of solutions of the platinum compounds in dimethylformamide (dioxane, dimethyl sulfoxide) with aqueous solutions of PVP afforded, depending upon the molecular weight of the PVP used (for the samples used in this study $\bar{M}_W \simeq 9{,}000$; 36,000; 140,000; 325,000), both water-soluble and water-insoluble complexes.

4-Substitutedcatecholato- and 4-Substitutedphthallato(1,2-diaminocyclohexane)-platinum(II) Compounds

These compounds were prepared from the appropriate ligands and cis-dinitrato(1,2-diaminocyclohexane)platinum(II) (19). The procedures listed below are typical of those used for the preparation of all the compounds reported herein.

cis-Dichloro(1,2-diaminocyclohexane)platinum(II). A solution of 6.01 g (0.015 mole) of potassium tetrachloroplatinate in 80 ml of 1.0 N aqueous hydrochloric acid was placed in a 250-ml, round-bottomed flask. 1,2-Diaminiocyclohexane (5.3 ml, 4.655 g, 0.0407 mole) was added in a single portion and the contents of the flask were swirled to effect through mixing. The flask was flushed with nitrogen, closed with a glass stopper and wrapped with aluminum foil to exclude light. The contents of the flask were held at 55° for 72 hr and then at 7° (refrigerator) for 10 hr. The yellow crystals which had formed were collected by filtration at reduced pressure, washed repeatedly with water and dried at reduced pressure over Drierite (4.71 g, 82% yield): ir (cm^{-1}, KBr) 3272 (s), 3199 (s) (N-H), 2947 (s), 2866 (m) (aliphatic C-H), 1564 (s), 757 (m) (Pt-Cl), 603 (w), 574 (w) (Pt-N); proton nmr (δ, DMSO-d_6) 0.68-3.24 (m-broad, 10H, cyclohexyl protons), 4.62-6.93 (m-broad, 4H, amino protons).

cis-Dinitrato(1,2-diaminocyclohexane)platinum(II). A solution of cis-dichloro-(1,2-diaminocyclohexane)platinum(II) (0.845 g, 0.0024 mole) and silver nitrate (0.425 g, 0.0022 mole) in 150 ml of water was placed in a 250-ml, round-bottomed flask containing a magnetic stirring bar. The flask was flushed with nitrogen, closed with a glass stopper and wrapped with aluminum foil to exclude light. The mixture was stirred at room temperature for two hours. The solution was freed of silver chloride which had precipitated by filtration at reduced pressure through a sintered glass filter. The absence of silver nitrate in the filtrate was confirmed by treating a small aliquot with 1.0 N aqueous hydrochloric acid solution; no precipitate was formed. The clear

colorless aqueous solution of cis-dinitrato(1,2-diaminocyclohexane)platinum(II) was utilized for the preparation of catecholato compounds containing the (1,2-diaminocyclohexane)platinum(II) moiety.

Catecholato(1,2-diaminocyclohexane)platinum(II). A portion of the aqueous solution of cis-dinitrato(1,2-diaminocyclohexane)platinum(II) previously prepared (40.0 ml, 0.0147 mmole/ml, 0.587 mmole) and 2.50 g (22.71 mmole) of catechol were placed in a 100-ml, round-bottomed flask. The flask was flushed with nitrogen, closed with a glass stopper and wrapped with aluminum foil to exclude light. The contents of the flask were maintained at 55° for 12 hr and then at 7° (refrigerator) for three hours. The dark crystalline solid which had formed was collected by filtration at reduced pressure, washed repeatedly with cold water and dried over Drierite at reduced pressure to afford the desired catecholato compound (0.135 g, 56.2% yield), m.p. > 250°: ir (cm^{-1}, KBr) 3226 (s-broad) (N-H), 2947 (s), 2864 (m) (aliphatic C-H), 1581 (s), 1483 (s) (aromatic nucleus), 1257 (s) (phenoxy C-O), 517 (w), 461 (w), 441 (w) (Pt-N), 363 (w), 322 (w) (Pt-O); proton nmr (δ, DMSO-d_6) 0.68-2.18 (m, 10H, cyclohexyl protons), 4.42-6.99 (m-broad, 4H, amino protons), 6.67 (m, 4H, aromatic protons).

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PRECLINICAL DEVELOPMENT AND FIRST CLINICAL STUDIES OF BUDOTITANE

B.K. Keppler, H. Bischoff, M.R. Berger, M.E. Heim, G. Reznik and D. Schmähl

INTRODUCTION

Cancer mortality is increasing on a world-wide scale, and thus many scientists, during the last decades, have aimed at developing new useful chemotherapeutic agents. These agents can be divided into those originating in organic chemistry and those coming from inorganic chemistry. Among the latter, cisplatin is the only substance to be widely established as anticancer agent in hospitals today. Cisplatin acts curatively only against testicular tumors. In addition, it has a lifespan-increasing effect along with minor cure rates in the case of ovarian cancer, cancer of the head and neck, and bladder tumors. In contrast to this, lung and gastrointestinal tumors, in particular colon tumors, are practically chemoresistant to cisplatin. Regardless of tumor regression achieved in some cases or others, it is not possible to obtain any major cure rates.

With respect to these findings, it is the most important aim of research in the field of cancer therapy to expand this rather narrow spectrum of indication by developing new antitumor-active metal complexes. The development of analogous platinum complexes has led to a little success only in decreasing toxicity, but it did not help to expand the spectrum of human tumors against

which these compounds are active. The only way to achieve this seems to be the synthesis of non-platinum complexes. Apart from a number of metal complexes which have shown antitumor activity in some preclinical models or others, there are only four non-platinum complexes which have been in clinical trials until this day. These are two germanium compounds - Germanium 132* and Spirogermanium** - as well as gallium complexes. The latter have had only some success during clinical studies. The fourth metal complex is budotitane*** (INN), which is the first transition metal complex to have reached clinical studies besides platinum compounds. Its development is described in the following.

SYNTHESIS OF BUDOTITANE AND RELATED COMPOUNDS

We synthesized typical representatives out of the class of bis-ß-diketonato metal complexes - M(ß-diketonato)$_2X_2$ - the general formula of which is shown in Fig. 1. These substances can usually be prepared by reaction of metal tetrahalide or metal tetraalkoxide with the corresponding ß-diketonate in an anhydrous organic solvent to form the dihalo- or dialkoxy-bis-ß-diketonato metal complexes, as demonstrated in the reaction scheme in Fig. 2. An exception is the synthesis of the corresponding molybdenum complex, which will be started with $MoCl_5$.

Structure-Activity Relation leading to the most active complex budotitane

Synthesis of about 200 of the complexes described in Fig. 1 led to our obtaining a strong structure-activity

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* Carboxyethylgermaniumsesquioxide
** N-(3-Dimethylaminopropyl)-2-aza-8,8-diethyl-8-germaspiro(4,5)decanedihydrochloride
*** Diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV)

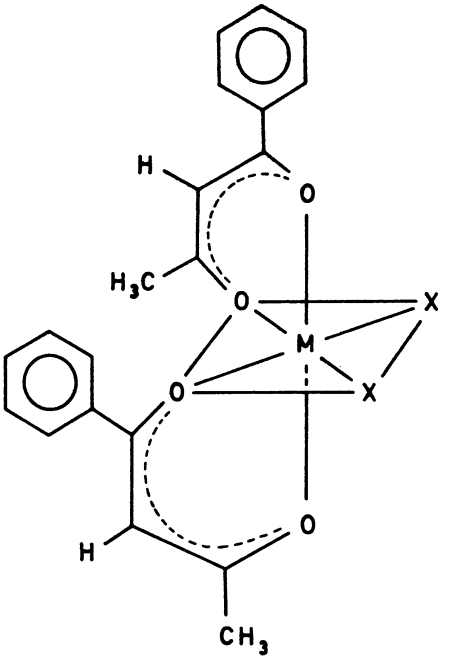


Fig. 1. General formula of the bis-ß-diketonato metal complexes - M(ß-diketonato)$_2$X$_2$; M = Ti, Zr, Hf, Mo, Sn, Ge.

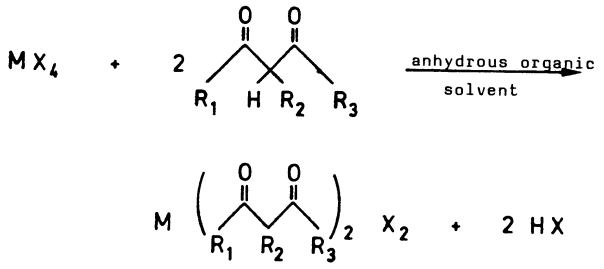
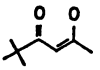
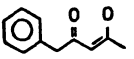
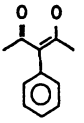
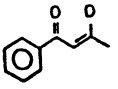


Fig. 2. General synthesis of M(diketonato)$_2$X$_2$ complexes X = Hal. or OR; R_1 - R_3 = organic moiety, M = Ti, Zr, Hf, Mo, Sn, Ge. In the case of Mo the reaction will start with $MoCl_5$.

relationship, which is illustrated with a few examples for the metal titanium in Fig. 3.

Fig. 3. Structure-Activity Relation of tumor-inhibiting bis-ß-diketonato titanium complexes*, $Ti(ß\text{-diketonato})_2X_2$

ß-diketonate	X	T/C (%)**	
	OEt	90 - 100	
	OEt	130 - 170	
	OEt	130 - 170	increasing activity
	OEt	200 - 250	
	OEt	300	budotitane

* assessed in the Sarcoma 180 Ascitic System with dose 0.2 mmol/kg; minimum and maximum values are given. The experiment is terminated when T/C values of 300 % are reached. The animals which are alive at this time are usually cured.

** T/C (%) = median survival time of treated animals versus control animals x 100.

These data demonstrate clearly that a planar aromatic ring is a necessary condition for antitumor activity in this class of anticancer agents. The last example in the figure above is the most active compound budotitane, which was selected for further studies and which is shown in Figure 4.

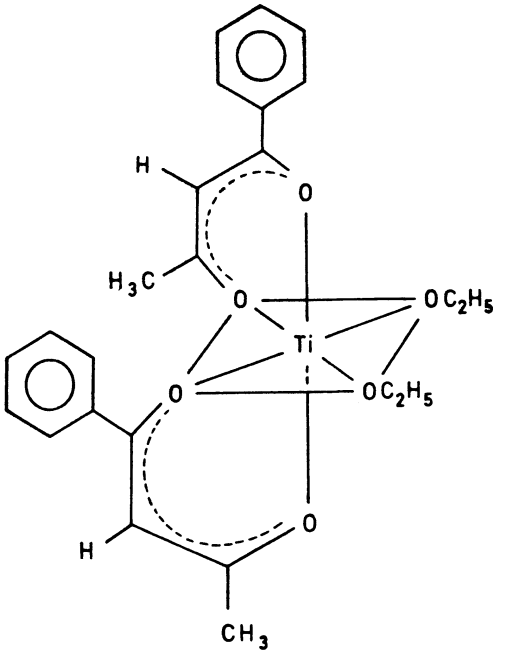


Fig. 4. Budotitane (INN), Diethoxybis(1-phenylbutane-1,-3-dionato)titanium(IV).

When using other metals, for which the same molecular structure could be obtained, decreasing activity was observed in the following order: titanium ≥ zirconium hafnium > molybdenum > tin > germanium.

ANTITUMOR ACTIVITY OF BUDOTITANE

Budotitane has been tested against several experimental tumor models until now, some of these are summarized in Fig. 5.

The last model in Fig. 5 will be explained more in detail. Here, adenocarcinomas of the colon are induced chemically in the gut of SD rats by intrarectal application of the carcinogen Acetoxymethylmethylnitrosamine. In their behaviour against chemotherapeutic agents and in their histology, these tumors clearly

reflect the clinical situation in colon cancer patients. They are completely resistant to cisplatin or agents such as cyclophosphamide but show some response to 5-fluorouracil. This applies also to the clinical situation. With respect to the high predictivity of this model, budotitane was tested against these tumors in comparison with cisplatin and 5-fluorouracil. The results are shown in Fig. 6.

Most astonishing was the superior activity of budotitane compared with the clinically established drug 5-fluorouracil in the experiment the results of which are illustrated in Fig. 6. As pointed out before, cisplatin is inactive in this model. The slightly tumor-stimulating effect of cisplatin might be explained by its immunosuppressive properties.

Fig. 5. Survey of the results of antitumor activity, which has been obtained with budotitane.

Tumor model	Evaluation parameter [1]	optimum T/C value[2] (%)
Sarcoma 180 ascitic tumor	ST	300* (1)
Sarcoma 180 tumor, subcutaneously growing	TW	0* (1)
Sarcoma 180 tumor, intramusculary growing	TW	30 (1)
Walker 256 Carcinosarcoma	ST	200 (2)
L 1210 Leukemia	ST	130 (1)
P 388 Leukemia	ST	130 (1)
Colon ascitic adenocarcinoma MAC 15A	ST	200* (3)
Autochthonous AMMN[3]-induced colorectal tumors	ST TW	146 20 (4)

[1] ST = survival time
TW = tumor weight

* 50 % or more cured

[2] T/C (%) = median tumor weight or survival time of treated animals versus control animals x 100.

[3] AMMN = Acetoxymethylmethylnitrosamine

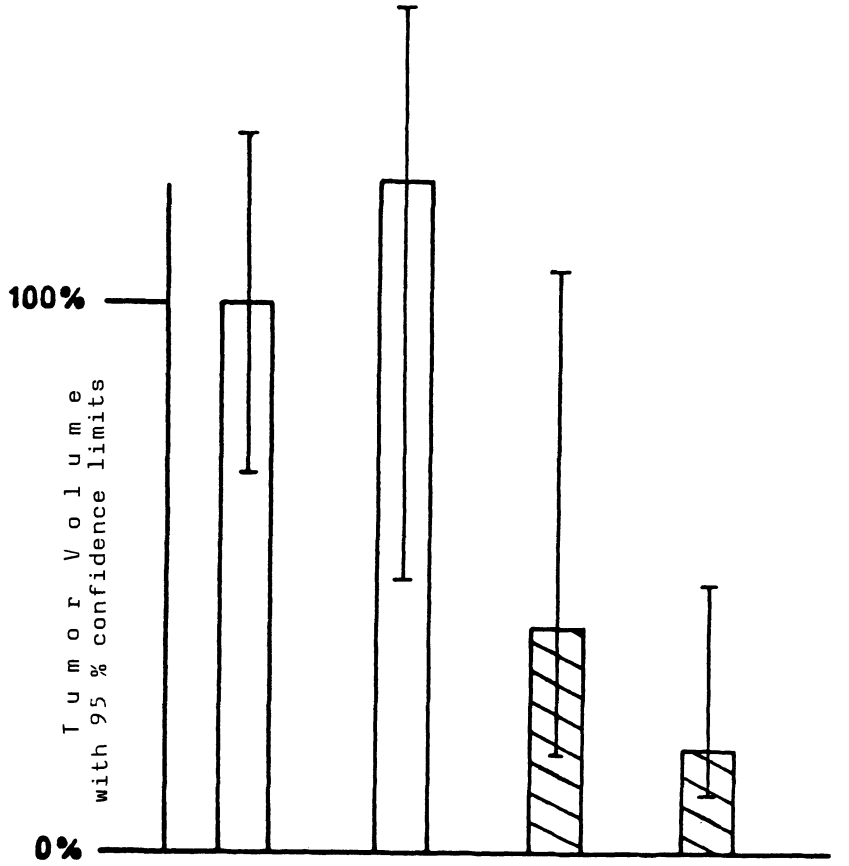


Fig. 6. Comparison of the effect of Cisplatin, 5-Fluorouracil and Budotitane on autochthonous colorectal tumors of the SD rat, induced by Acetoxymethylmethylnitrosamine. (According to data from Ref. 4).

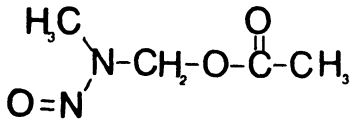


Fig. 7. Acetoxymethylmethylnitrosamine (AMMN)

TOXICITY OF BUDOTITANE

Lethality Study. 10 male and 10 female rats per dose, single intravenous administration.

Dose (mg/kg)	Mortality
0 (untreated)	0/20
0 (solubilizer)	0/20
20	0/20
26	0/20
33	0/20
42	0/20
55	4/20
70	19/20
90	20/20

Spontaneously dead animals, macroscopic findings: hydrothorax, icterus, hemorrhage in glandular stomach, pronounced swelling of glandular stomach mucosa. Histology: liver - necrobiotic foci, nucleated erythrocytes; kidneys - protein casts, hyaline thrombi in glomeruli. Animals killed at end of study: stomach - severe elevation of mucosa, hyperkeratosis of the border fold, ulcers from 26 mg/kg; spleen - cell proliferation in red pulp; liver - at 55 and 42 mg/kg pigment deposits in Kupffer's cells.

Single dose toxicity (main study). 12 male and 12 female rats per dose.

Dose (mg/kg)	Mortality
0 (untreated)	0/24
0 (solubilizer)	0/24
10	0/24
35	0/24
60	7/24

Clinical findings: 60 mg/kg - reduced motility, piloerection and icterus in all animals. Hematology: 60 mg/kg on day 4: prolonged thromboplastin time, increased reticulocytes, leukocytes nRBC's and increase in the proportion of the polynuclear cells, decreased hemoglobin, hematocrit, thrombocytes and small lymphocytes. Hematocrit value was slightly decreased in all treated animals on day 29.

Clinical chemistry: 60 mg/kg - marked elevations in urea, AP, ALAT and ASAT. Autopsy: in all dose groups, hemorrhage in glandular stomach. Histology: multiple liver necroses at 60 mg/kg in 9/12 animals. Marked activation of the hematopoetic system in the bone marrow with extramedullar foci in liver and spleen at 60 mg/kg and slight activation at 35 mg/kg on day 4 of autopsy.

Multiple dose toxicity. 10 male and 10 female rats per dose, 2 doses per week for 12 weeks.

Dose (mg/kg)	Mortality
0 (solubilizer)	0/20
4.5	0/20
9	0/20
18	0/20

Clinical findings: piloerection, reduced motility, hunch-backed sitting at 18 mg/kg. Hematology: Day 79, depression in red blood count in the 18 mg/kg group. Clinical chemistry: Total proteins, albumin, globulins and Ca reduced on Days 9, 44 and 79 at 9 and 18 mg/kg, Ca also reduced at 4.5 mg/kg on Days 44 and 79. AP and ALAT were elevated at all doses and creatinine and urea at 18 mg/kg on Day 79. Histology: Dose-dependent pigment deposits in liver, lymph nodes and spleen. Occasional hydropic swelling in the liver, increases in inflammatory reactions and liver cell dissociation.

Conclusions from toxicological studies. In therapeutic doses, i.e. doses between 5 and 10 mg/kg applied twice per week over several weeks, mild hepatotoxicity seems to be the only undesirable side effect. If these findings are confirmed in clinical studies, this will be an acceptable adverse effect, especially for an antitumor agent.

CLINICAL STUDIES

On account of budotitane having shown a good antitumor activity in preclinical experiments, in particular against colorectal tumors, and due to its relatively mild toxicity in therapeutic doses, as described above, a clinical phase I study was started.

Selection of patients. 1. Patients with malignant tumors that do not seem curable with standard therapies. 2. Patients between the age of 18 and 75. 3. Karnofsky Index ≥ 40 %. 4. Sufficiently intact functioning of liver, kidneys, and bone marrow. 5. Patients not having received any chemotherapy during the last four weeks prior to the beginning of treatment. 6. An estimated life expectancy of at least 3 months.

Aims of the study. 1. Determination of the maximum tolerated dose (MTD). 2. Toleration of repeated application (2 x/week) over 4 weeks. 3. Pharmakokinetic investigations.

The single dose trial was started with a safe initial dose of 1 mg/kg. Three patients were treated with each dose. All clinical findings, hematological parameters and clinical chemistry values were carefully monitored. If there was no stage II toxicity according to WHO criteria, the next higher dose was tested. Dose escalation was done according to the Gottlieb method. The following doses have been employed so far: 1, 2, 4, 6, 9, 14, 21 mg/kg. Beginning with 9 mg/kg, the patients complained about impairment of the sense of taste shortly after the infusion, but this effect disappeared within a few hours. At a dose of 21 mg/kg, a slight increase in liver enzymes and in LDH was observed. In two out of three patients there was a severe increase in creatinine, so that this dose may be considered the MTD. A study on repeated application is now being started. A multicenter phase II study under the auspices of the AIO is scheduled for next year.

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ANTITUMOR ACTIVITY OF A NEW PLATINUM CYTOSTATIC, DWA2114R

M. Koizumi, M. Honda, K. Morikawa, K. Endoh, T. Matsumoto, K. Akamatsu and H. Matsui

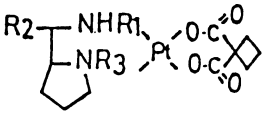
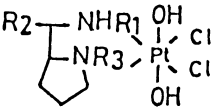
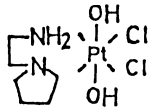
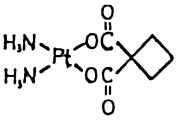
Antitumor platinum complexes containing aminoalkylpyrrolidine derivatives were synthesized. Antitumor activities of these platinum complexes were tested in vivo against colon 26 carcinoma. Among them, 2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-platinum (II) (1a) exhibited the highest activity. As compound 1a had an assymmetric carbon, the stereo isomers S-form (1aS) and R-form (1aR) of 1a were synthesized and were examined the antitumor activity and the toxicity of them. It was shown that the antitumor activities of these compound were virtually same, whereas the nephrotoxicity was only shown in 1aS.

On the basis of these observation, 1aR (DWA2114R) was selected for further pharmacological and clinical evaluation.

Cis-diamminedichloroplatinum (II) (CDDP) is the potent antitumor agent but owing to the serious nephrotoxicity, its effective use in cancer chemotherapy has been limited.(1,2) A great number of attempts have been made in many countries to obtain active but less toxic antitumor platinum complexes since the discovery of antitumor activity of CDDP by B. Rosenberg(3) in 1969.

For this purpose, the unsymmetrical diamines were given great attention as carrier ligands and platinum complexes(4) containing aminoalkylpyrrolidine derivatives were synthesized and tested for their antitumor activity against colon 26 carcinoma (sc-ip).(6) These compounds were shown to have a tendency that the substitution of the aminopyrrolidine derivatives reduced the antitumor activity and alternately Ia was screened as most active compound (Table 1).

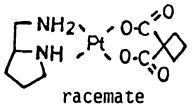
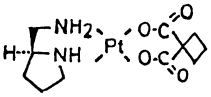
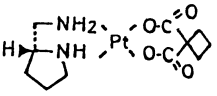
Table 1. Antitumor Activity of Platinum Complexes Containing Aminoalkylpyrrolidine Derivatives Against Colon 26 Carcinoma (sc-ip)

Platinum Complex	No	R1	R2	R3	GIR (%) 40 mg/kg
 1	1a	H	H	H	95
	1b	Me	H	H	-6
	1c	H	Me	H	54
	1d	H	H	Me	11
	1e	H	H	Et	—
 2	2a	H	H	H	75
	2b	H	H	H	37
	2c	H	Me	H	48
	2d	H	H	Me	44
	2e	H	H	Et	67
	3				56
	CBDCA				57

Animal: CDF1/Crj mice, male, 6 w, n=5-6
Tumor: colon 26 carcinoma, 1-2 mm^3 fragment, sc
Treatment: on day 4, 1 shot, ip
Evaluate: on day 14, measurement of tumor weight
GIR (%) = (C - T)/C x 100
(T: tumor weight in treated animals)
(C: tumor weight in control animals)

As compound 1a had an assymmetric carbon, we synthesized the stereo isomers of 1a and compared the antitumor activity and the toxicity of them. S-(+)-2-aminomethylpyrrolidine and R-(-)-2-aminomethylpyrrolidine which were optically active carrier ligand of 1aS and 1aR, were synthesized for several steps from S-(-)-proline and R-(+)-proline, respectively.(7,8) The structure and the physical data of 1a, 1aS and 1aR are shown in Table 2.

Table 2. Structure and Physical Data of 1a, 1aS and 1aR

Compound	Structure	mp (°C)	IR $\overset{KBr}{max}$ cm^{-1}	$[\alpha]_D^{20}$
1a	 racemate	215-220 (dec.)	3190, 3100 (N-H) 1635, 1590 (C=)	—
1aS		240-255 (dec.)	3190, 3100 (N-H) 1635, 1590 (C=O)	+39° (c=0.4, H_2O)
1aR		248-257 (dec.)	3190, 3100 (N-H) 1635, 1590 (C=O)	-40° (c=0.4, H_2O)

Antitumor activity of these three platinum complexes against various tumors are shown in Table 3.

Table 3. Comparison of Antitumor Activities of 1a, 1aS, and 1aR Against Various Tumors

Tumor	Treatment	Antitumor Activity GIR (%) 1a	1aS	1aR
Colon 26 (sc)	40 mg/kg x 1, ip (day 4)	72	80	69
Colon 38 (sc)	40 mg/kg x 3, iv (day 6, 8, 10)	88	94	75
B16 (sc)	40 mg/kg x 3, iv (day 7, 9, 11)	72	85	62
Ca 755 (sc)	10 mg/kg x 3, iv (day 4, 6, 8)	87	97	91
		ILS (%)		
L1210 (ip)	40 mg/kg x 1, ip (day 1)	115	131	96
P388 (ip)	40 mg/kg x 1, ip (day 1)	90	107	101

ILS (%) = (T/C - 1) x 100
(T: life span in treated animals)
(C: life span in control animals)

Table 3 shows that the order of the activity is 1aS > 1a > 1aR, but there is not so significant difference between them.

Then we compared the toxicity of 1a, 1aS and 1aR. Table 4 shows the acute toxicity of these three platinum complexes.

Table 4. Acute Toxicity of 1a, 1aS and 1aR in BDF_1 Mice (single ip injection)

Compound	LD_{50} (mg/kg)
1a	118 ± 6
1aS	109 ± 9
1aR	123 ± 9

95% confidence limit

The order of the acute toxicity is 1aS > 1a > 1aR, but there is not so great difference between them. Next, we compared the nephrotoxicity of 1a, 1aS and 1aR at MTD (maximum tolerated dose) by estimating BUN, urinary protein (UP) and urinary sugar (US) as an index of nephrotoxicity. it was shown that the remarkable increase of BUN, UP and US was observed in 1aS, while no increase in 1aR (DWA2114R).

On the basis of these observation, we selected DWA2114R which is now in progress for a phase I trial.

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BIS(URACIL-)-COMPLEXES OF CISPLATIN: PREPARATION, PROTONATION AND METAL BINDING

O. Krizanovic and B. Lippert

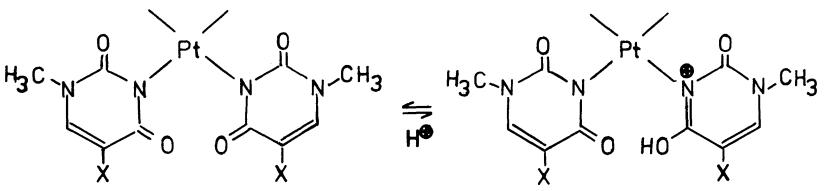
INTRODUCTION

Bis(1-methylthyminato-N(3)cis-diammineplatinum-(II)),cis-$(NH_3)_2Pt(1\text{-meT})_2$, is a model compound for a hypothetical interaction of the antitumor agent cis-$Pt(NH_3)_2Cl_2$ with two thymine bases of DNA. Protonation of the N(3) platinated ligand at O(4) leads to Pt-complexes containing the rare imino tautomer of this nucleobase /1/.

It has been the purpose of this work to study a series of cis-$(NH_3)_2PtL_2$ complexes containing L=5-substituted 1-methyluracils and L=5,6-dihydro-1-methyluracil and to estimate the effect of ligand modification on the basicity (H^+-affinity) of O(4).

RESULTS

Protonation of cis-$(NH_3)_2PtL_2$. Complexes of composition cis-$(NH_3)_2PtL_2$ with L=1-methyluracil (1-meU),1, 1-methylthymine (1-meT),2, 5-chloro-1-methyluracil (5-Cl-1-meU),3, 5-nitro-1-methyluracil (5-NO_2-1-meU),4, and 5,6-dihydro-1-methyluracil (5,6-dihydro-1-meU),5, were prepared and protonation according to



was determined by use of UV-spectroscopy (1 - 4) and ^{1}H-NMR-spectroscopy (5) respectively. UV-spectra recorded in the pH-range 5,95 - 0,3 showed isosbestic behaviour, consistent with the above equilibrium.
A representative example is given in figure 1 and 2.

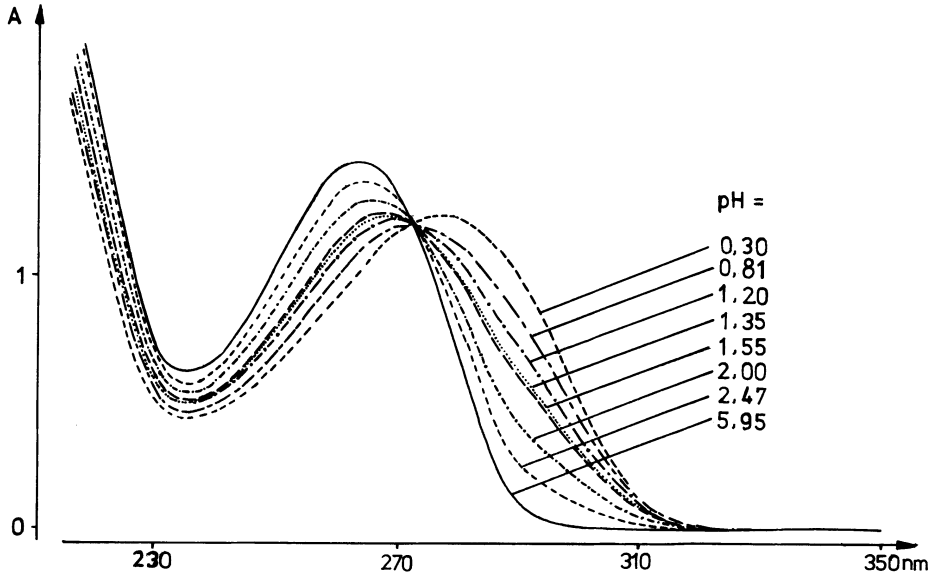


Fig.1 : UV-spectra of 1 in the pH-range 5,95-0,3

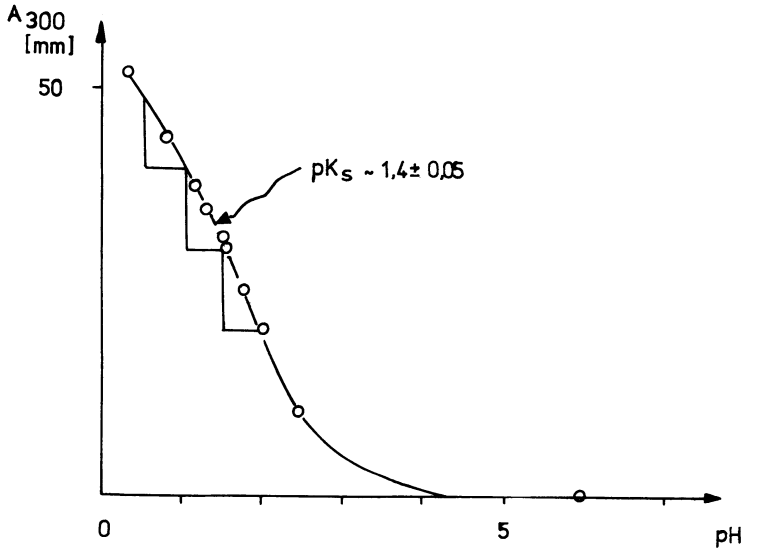


Fig.2 : Extinctions at 300 nm at different pH-values

From a plot of A_{300nm} vs pH, a pK_a of 1,4 ± 0,05 was obtained for 1. Similarly, 2 gave a value of 2,0 ± 0,05 in good agreement with previous results /1/. Protonation of 3 was incomplete at pH 0 (pK_a estimated as -1) and no protonation was detected for 4. From the pH-dependence of the ^{1}H-NMR chemical shifts of 5 in D_2O, a pK_a of ca 0 was estimated.
In the following table, pK_a-values of protonated bases LH^+ and the corresponding species cis-$[(NH_3)_2PtL(LH)]^+$ are compared.

	free ligand	N(3) platinated ligand	$\triangle pK_a$
1-meU	≈ -3,0	1,4	4,4
1-meT	≈ -3,0	2,0	5,0
5-Cl-1-meU	< -5,0	≈ -1,0	> 4,0
5,6-dihydro-1-meU	-4,5	≈ 0	4,5

Values for LH^+ were taken from the literature /2,3,4/ and estimated from related systems, respectively. Only average values are given because of the dependence on solvent and acidity functions used. Differences in pK_a-values appear to be constant (4-5-log units) and independant of the substituent of the nucleobase.

Di-and Trinuclear Complexes Derived from 3. The increase in ligand basicity in cis-$(NH_3)_2PtL_2$, a con-

sequence of substitution of N(3)H by Pt(II), is also evident from the high tendency to form di- and multinuclear complexes. Reaction of 3 with cis-$[(NH_3)_2Pt(H_2O)_2]^{2+}$, Cu^{2+}, and $[PtCl_4]^{2-}$ led to complexes of composition cis-$[(NH_3)_2Pt(5\text{-}Cl\text{-}1\text{-}meU)_2Pt(NH_3)_2](NO_3)_2$, 6, $[(NH_3)_4Pt_2(5\text{-}Cl\text{-}1\text{-}meU)_4Cu](NO_3)_2 \cdot 7{,}5\ H_2O$, 7, and cis-$(NH_3)_2Pt(5\text{-}Cl\text{-}1\text{-}meU)_2PtCl_2 \cdot 0{,}5\ H_2O$ respectively. 6 and 7 are the analogues of the corresponding 1-meU-complexes which were characterized by X-ray crystallography /5,6/. 6-8 were identified by elemental analysis and IR-spectroscopy.

EXPERIMENTAL

Preparation. 1 and 2 were prepared as previously described. 3 and 4 were obtained analogously. 5 was prepared similarly, but isolation proved difficult because of high solubility. Addition of HNO_3 to a concentrated solution of 5 (pH 0,5 had to be reached) gave $[(NH_3)_2PtL(LH)] \cdot (NO_3) \cdot 2H_2O$, 5*, as colorless crystal needles.

Analysis	for 3:					
found:	C	19,92%	H	2,40%	N	13,79%
calcd:	C	19,94%	H	2,34%	N	13,95%
	for 4:					
found:	C	20,44%	H	2,40%	N	19,77%
calcd:	C	21,09%	H	2,48%	N	19,68%
	for 5*:					
found:	C	20,52%	H	3,57%	N	17,20%
calcd:	C	20,62%	H	3,63%	N	16,83%
	for 6:					
found:	C	13,40%	H	2,29%	N	15,29%
calcd:	C	13,32%	H	2,24%	N	15,54%

	for 7:					
found:	C	14,45%	H	2,38%	N	9,98%
calcd:	C	14,59%	H	1,83%	N	10,20%
	for 8:					
found:	C	17.69%	H	2.71%	N	14,55%
calcd:	C	17,70%	H	3,19%	N	14.47%

UV-measurements were carried out on a Perkin-Elmer-Spectrophotometer 555, ^{1}H-NMR-spectra (D_2O, pD uncorrected, NMe_4^+ as internal reference) on a Varian EM 360 (60 MHz), a Varian EM 390 (90 MHz) and Bruker WM 250 (250 MHz). IR-spectra were taken on a Perkin Elmer Spectrometer, model 577.

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CIS-DIPHOSPHINE PLATINUM(II) COMPLEXES WITH PYRIMYDIL NUCLEOSIDES: SYNTHESIS, CHARACTERIZATION AND STRUCTURAL STUDIES

B. Longato, B. Corain, G.M. Bonora, G. Valle and G. Pilloni

INTRODUCTION

We have recently reported on the unusually high reactivity of the cation cis - $[L_2PtS_2]^{2+}$ (L_2 = 1,1'-Bis(diphenylphosphino) ferrocene-P,P'; S=Dimethylformamide (DMF), Dimethyl sulfoxide (DMSO)) toward thymidines and uridines (1). The synthesis and spectral characterization of the adducts $[L_2Pt(X)(nucleoside(-H))]^{1+,0}$ (X=DMF, DMSO, Cl^-) were also reported (2).

We report here on the reactivity of the complex cis-$[L_2Pt(\mu\text{-}OH)]_2^{2+}$, **1**, (3), with 3',5'-diacetylthymidine, $Ac_2(dT)$, and deoxycytidine, dC, as well as with the corresponding nucleobase 1-methyl-thymine, 1-Me-Ty. The aim of this work was the preparation and characterization of mixed bisnucleosido complexes which could be related to the interaction of the $\{cis\text{-}L_2Pt^{II}\}$ moiety with two nucleic acid components. This moiety appears to be able to coordinate both thymidine and cytidine affording adducts of the type $[L_2Pt(thymidinate)(cytidine)]^+$.

The preliminary X-ray single crystal structure of $[L_2Pt(dC)(1\text{-}Me\text{-}Ty(-H))](BF_4)\ 0.5Me_2CO$ shows that cytidine exhibits an unprecedented binding mode in platinum(II) chemistry.

MATERIALS AND METHODS

All solvents were dried over molecular sieves. Complex $[L_2Pt(\mu\text{-}OH)]_2(BF_4)_2$, **1**, was prepared as previously reported (2,3). ^1H and ^{31}P NMR spectra were recorded on a JEOL 90Q spectrometer at 27°C and were referenced to internal $SiMe_4$ and external H_3PO_4, respectively. 1-Methyl-thymine was obtained from Sigma Chemicals, dC H_2O from Fluka, and were used without further purification. The nucleoside thymidine (Fluka) was acetylated according to the procedure previously reported (4).

$[L_2Pt(dC)(Ac_2(dT)(-H))]BF_4$, Isomer A, 3.

A solution of **1**.(200 mg, 0.117 mmol) and $Ac_2(dT)$ (77 mg, 0.234 mmol) in DMF (5 ml) was stirred at room temperature (r.t.) for two days. dC·H_2O (58 mg, 0.234 mmol) was then added to the resulting solution of **2** and the reaction mixture stirred a few min. After vacuum evaporation of the solvent, the residuum was dissolved in $CHCl_3$; addition of Et_2O gave an oil which, upon freezing at 77 K and warming to r.t., was converted into a powdered solid and collected by filtration. The purification by dissolution in $CHCl_3$ and precipitation with n-hexane afforded 263 mg of pure **3** as yellow microcrystals (yield 80%). Anal. Calc. for $C_{57}H_{60}N_5O_{12}P_2BF_4FePt$: C,48.66;H,4.15;N,4.98. Found: C,47.88;H,4.20;N,4.78.

$[L_2Pt(dC)(Ac_2(dT)(-H))]BF_4 \cdot CH_2Cl_2$, Isomer B, 4.

150 mg of **3** were dissolved in CH_2Cl_2 (10 ml). After 24 hr the solution was allowed to concentrate by slow evaporation. A yellow microcrystalline precipitate was then formed, collected by filtration and dried under vacuum. 85 mg of pure **4** (yield 53%) were obtained. Anal. Calcd. for $C_{58}H_{62}N_5O_{12}P_2BCl_2F_4FePt$: C,46.70;H,4.12;N,4.69. Found: C,46.90;H,4.12;N,4.55.

$[L_2Pt(dC)(1\text{-}Me\text{-}Ty)(-H))]BF_4 \cdot 2CHCl_3$, 5.

A solution of **1** (300 mg, 0.176 mmol) and dC·H_2O (86 mg, 0.352 mmol) in DMF (5 ml) was stirred at room temperature for 3 hr. 1-Me-Ty

(49 mg, 0.352 mmol) was then added and the reaction mixture stirred at r.t. for 2 days. The solvent was vacuum evaporated and the residuum dissolved in a mixture of CH_2Cl_2 (2 ml) and $CHCl_3$ (10 ml). Upon slow evaporation at r.t.,yellow crystals of **5** were formed (390 mg, yield 77%). Anal. Calcd. for $C_{51}H_{51}N_5O_6P_2BCl_6F_4FePt$: C,42.50;H,3.57;N,4.86. Found: C,42.57;H,3.51;N,4.67. ^{31}P NMR in DMF: δ10.9 (doublet, relative intensity 1.0, J_{Pt-P} 3559 Hz, J_{P-P} 18.2 Hz); 3.21 (doublet, relative intensity 0.5, J_{Pt-P} 3315 Hz, J_{P-P} 18.2 Hz); 3.08 (doublet, relative intensity 0.5, J_{Pt-P} 3315 Hz, J_{P-P} 18.3 Hz).

Crystallography of $[L_2Pt(dC)(1\text{-Me-Ty}(-H))]BF_4$, 5, Isomer B.

Suitable crystals of **5** were grown from acetone, upon slow evaporation and analyzed as $[L_2Pt(dC)(1\text{-Me-Ty}(-H))]BF_4 \cdot 0.5(CH_3)_2CO$. Diffraction intensities were collected on a Philips PW 100 four circles diffractometer with graphite monochromated MoKα radiation (λ=0.7107 Å). **5** crystallizes in the monoclinic system, space group $P2_1$, with a=14.292(2) Å, b=28.811(3) Å, c=13.212(2) Å, β=108.3(1)°. Z=4; D_c=1.58 g cm^{-3}. A total of 8606 reflections were collected ($2° \leq 2\vartheta \leq 50°$; ϑ-2ϑ scans), of which 6898 have $I \geq 2\sigma(I)$. The structure was solved by the heavy metal method and was refined by full matrix procedure (SHELX program). The final R factor was 0.112 (unit weights).

RESULTS AND DISCUSSION

Synthesis of $[L_2Pt(dC)(Ac_2(dT)(-H))]BF_4$: Isomer A, 3, and B, 4.

By reacting complex **1** with $Ac_2(dT)$ and dC in DMSO or DMF, two isomeric species, i.e.,**3** and **4**, are obtained, depending on the sequence in the addition of the biomolecules as depicted in Scheme 1. Reactions leading to isomers A and B were monitored by ^{31}P NMR. Intermediate **2** is formed quantitatively (2) and it is instantaneously converted into **3** with quantitative yield by reaction with the stoichiometric amount of dC. The ^{31}P NMR data are collected

in Table 1. The two couples of resonances observed in DMF are attributed to the phosphorus atoms trans to the thymidinate ligand (2) (δ3.60, doublet with J_{P-P} 18 Hz and J_{Pt-P} 3521 Hz) and to the cytidine one (δ-1.20, doublet with J_{P-P} 18 Hz and J_{Pt-P} 3725 Hz), respectively. This attribution stems from the analogy with the relevant $^{195}Pt-^{31}P$ coupling constant observed in intermediate **2** (J_{Pt-P}=3403 Hz, (2)) and it fits with the expected higher basicity of dC with respect to the coordinated solvent (J_{Pt-P}=3725 vs. 4394 Hz).

Addition of dC to **1** in 1:1 molar ratio, leads to the incomplete disappearance of complex **1** and to the instantaneous appearance of two sets of signals at δ-1.98 (doublet, J_{Pt-P} 3750 Hz, J_{P-P} 17 Hz), 9.86 (doublet, J_{Pt-P} 3255 Hz, J_{P-P} 17 Hz) and -0.59 (doublet, J_{Pt-P} 3750, J_{P-P} 18.3 Hz), 10.65 (doublet, J_{Pt-P} 3238 Hz, J_{P-P} 18.3 Hz). The relative intensities of the two sets are 2.6:1 in favour of the first one. The data presently available do not make possible an unambigous attribution of the observed resonance pattern to the involved intermediates. However, as a matter of fact, the addition of the stoichiometric amount of Ac_2(dT) to this reaction mixture affords the gradual and quantitative formation of **4**. The ^{31}P NMR spectrum of this species (Table 1) displays two doublets with fairly different coupling constants, the highest of which being quite similar to that seen in the spectrum of **3** and attributed to the phosphorus atom trans to the thymidinate ligand. On the contrary, the resonance at δ 3.1 is characterized by a J_{Pt-P} (3347 Hz) quite lower than that attributed to the phosphorus atom trans to N3 bonded cytidine for isomer **3**. This noticeable spectral difference has to be due to a substantial change in the nature of the binding site utilized by cytidine. In fact, the decrease of this coupling constant can be justified by the involvement of the exocyclic amino group, in its deprotonated form (Fig.1), which is made possible by proton transfer to either N3 or O2 of cytidine. The occurence of an intramolecular proton transfer is

proved by the monocationic nature of complex **4** and the location of the transferred proton at N3 is suggested by a broad resonance at δ 10.8 (DMSO-d_6) in the ^{1}H NMR spectrum of **4**. Finally, our structural proposal stands on further related data described in the next Section. It is worth underlining that isomer A tourns out to be thermodynamically unstable with respect to isomer B into which it is quantitatively converted in $CHCl_3$ or CH_2Cl_2 in ca. 24 hr. Moreover, in these solvents both **3** and **4** exist as two configurational isomers arising from the hindered rotation of the ribose residue around N1-C1' bond of the nucleosides (5).

Synthesis and Structure of [L_2Pt(dC)(1-Methyl-Thyminate)] BF_4, 5, (Isomer B).

The impossibility of obtaining single crystals of complex **4** prompted us to prepare a closely related species by replacement of Ac_2(dT) with the corresponding 1-methyl substituted nucleobase, 1-Me-Ty. By following route b) the title complex was formed in DMF and isolated as yellow crystals in good yield. The ^{31}P NMR spectrum (see Material and Methods) displays parameters almost identical with those given by **4**. Along route a), a complex related to **3** was also obtained (6).

X-ray single crystal analysis of complex **5** gave, so far, a residual factor equal to 0.112. On the basis of this result, precise bond distances and angles are not yet available, but the location of the atoms is nevertheless quite established. The structure contains monomeric moieties with the chelating diphosphine, N3 bonded 1-methylthyminate and the cytidine ligand, which appears coordinated through the deprotonated exocyclic amino group. In fact, although the location of the transferred proton is not yet established, the observed Pt-N4-C4 angle is 120.1(28)° (7).

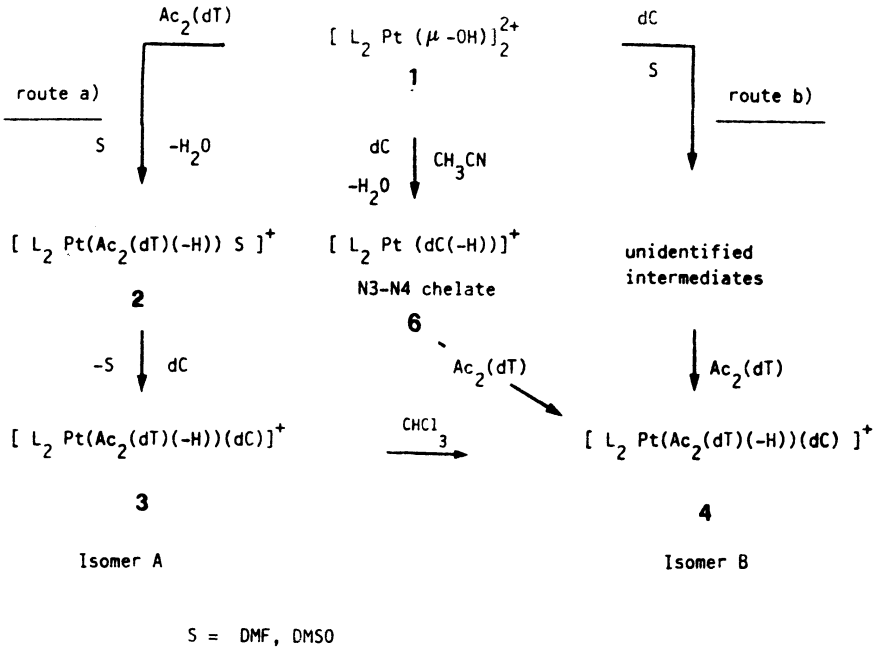
Mechanism of the Conversion of 3 (Isomer A) into 4 (Isomer B): Synthesis of N3-N4 Chelate $[L_2Pt(dC(-H))]BF_4$, 6.

The mechanism of the conversion of **3** into **4** could conceivably involve a pentacoordinated transition state in which the metal undergoes coordination by both the exocyclic nitrogen and N3 of cytidine. Such coordination mode has been firmly established in a Pt(IV) complex with 1-methyl-cytosine (8) and also proposed on the basis of kinetic evidences in the reaction of cytidine with $[(dien)Pd(OH_2)]^{2+}$ (9). This reaction is proposed to involve: i)preliminary coordination through N3; ii)induced deprotonation of the amino residue; iii)metal coordination of the so formed NH^- group with concomitant release of the previously coordinated N3 binding site. We believe that a similar mechanism is operative in the present case and a strong support to the involvement of a reaction intermediate, in which the NH^- and N3 sites are simultaneously involved, is given by the synthesis and reactivity of complex **6**. In fact, by reacting **1** with cytidine in CD_3CN (Scheme 1), under anhydrous conditions, complex **6** was quantitatively formed, characterized by ^{31}P NMR and isolated as BF_4^- salt (6). As expected, addition of $Ac_2(dT)$ to **6** in CD_3CN afforded quantitatively complex **5** (isomer B).

CONCLUSIONS

The results described in this paper extend to platinum(II) the very unusual coordination behavior of cytidine recently disclosed for platinum(IV) (10). Again, the observed utilization of the exocyclic nitrogen as binding site is made possible by an intramolecular proton transfer and this bonding mode requires to be considered in the bioinorganic chemistry of this DNA component.

Scheme 1. Different routes to mixed bisnucleosido cis-platinum (II) complexes.



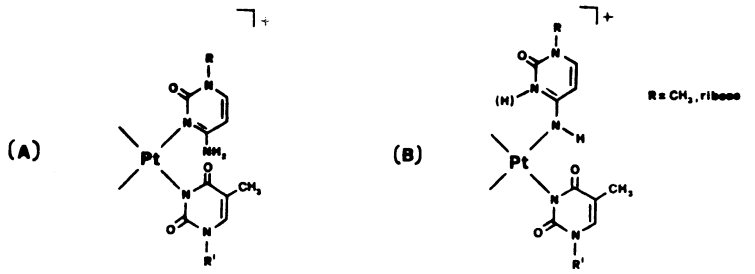


Fig.1. Proposed structures for adducts 3 and 4 formed along routes a) and b).

	Solvent	δ	$J_{^{195}Pt-^{31}P}$	$J_{^{31}P-^{31}P}$	Rel.Int.
Route a)	$CDCl_3$	2.90	3505	18	1.0
3		0.57	3720	18	0.5
		0.12	3720	18	0.5
	DMF	3.60	3521	18	1.0
		-1.20	3725	18	1.0
Route b)	$CDCl_3$	11.20	3602	18	1.0
4		4.36	3349	18	0.5
		4.29	3349	18	0.5
	$DMSO-d_6$	10.80	3526	18	1.0
		3.10	3347	18	1.0

Table 1. ^{31}P NMR data for $[L_2Pt(dC)(Ac_2(dT)(-H))]BF_4$ complexes, **3** and **4**, in different solvents at 27° C. Chemical shift (δ) values in ppm and coupling constant (J) values in Hz.

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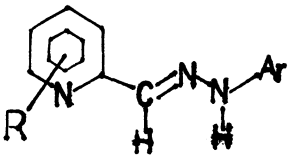
ANTITUMOR PROPERTIES OF METAL (II) COMPLEXES OF 2-PYRIDINE CARBOXALDEHYDE 2'-PYRIDYLHYDRAZONES AND RELATED COMPOUNDS

M. Mohan

INTRODUCTION

Aroylhydrazones have been shown to exhibit significant inhibiting effect on DNA synthesis and cell growth in a number of human and rodent cell lines in culture (1). A copper (II) complex of the most potent of the chelators, salicylaldehyde benzoylhydrazone (SBH), has greater inhibitory activity than does SBH itself (2). These interesting results stimulated us to study the antitumour activity of related ligands and their metal chelates. This paper describes the synthesis, characterization and antitumour activity of 2-pyridine carboxaldehyde 2'-pyridylhydrazone and related ligands I-VII and their metal (II) chelates.



i. R = H, Ar = C_5H_4N (PCPH);

ii. R = CH_3, Ar = C_5H_4N (6-MePCPH);

iii. R = H, Ar = 3'-CH_3, C_5H_3N (3'-MePCPH);

iv. R = H, Ar = 4'-CH_3, C_5H_3N (4'-MePCPH);

v. R = H, Ar = 5'-CH_3, C_5H_3N (5'-MePCPH);

vi. R = H, Ar = 6'-CH_3, C_5H_3N (6'-MePCPH); and

vii. R = H, Ar = C_9H_6N (PCQH).

MATERIALS AND METHODS

The ligands I-VI were prepared according to the reported methods (3). All other chemicals and solvents were Reagent grade or equivalent. 2-Pyridine carboxaldehyde 2´-quinolylhydrazone was purchased from Aldrich Chemical Co., Wisconsin and was used as such.

Preparation of Metal (II) Complexes

All the metal (II) chelates were prepared by the following general method. A hot solution of ligand (1 mmol) in 95% ethanol (25 mL) was added to a hot solution of metal (II) salt (1 mmol) in ethanol (15 mL) or an aqueous solution of K_2PtCl_4(0.415g, 1 mmol) and the resulting solution mixture was heated under reflux for 15-30 min. The boiling solution, on cooling to room temperature, afforded fine crystalline solid which were filtered off, washed with ethanol and diethylether and dried over P_2O_5 under vacuum.

Physical Measurements

The physical measurements were carried out according to the reported procedure (4). The analytical results for these elements were within $\pm$ 0.4% of the theoretical values.

Evaluation of Antitumour Activity

The antitumour activity of the ligands I-VII and their metal (II) chelates was determined according to the reported procedures (4,5).

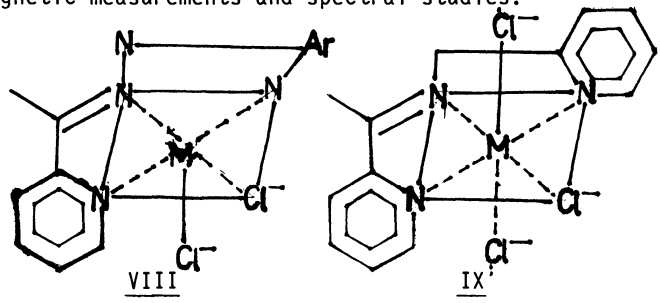
Incorporation of Radioactive Precursors into DNA, RNA & Protein

The effect of Cu(PCPH) Cl on macromolecular synthesis of DNA, RNA and protein was determined by the reported procedure (6).

RESULTS

Chemistry

The products that were obtained from Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Pt(II) are monoligand complexes that have been assigned the distorted five-coordinate (square-pyramidal) structures represented by VIII, except Ni(PCPH) Cl_2 which is chloro-bridged, high-spin, trans-distorted six-coordinate complex IX. This conclusion is based on micro-analytical data, conductance data, magnetic measurements and spectral studies.



Biological activity and discussion

The ligands I-VII and their metal (II) chelates were tested for antitumour activity against P 388 lymphocytic leukemia in mice by the National Cancer Institute, Bethesda, MD. The results indicated that 2-pyridine carboxaldehyde 2´-pyridylhydrazone, I, (PCPH) was a inhibitor of P 388, having T/C, % value of 138 at a dose level of 60 mg/kg. Substitution in the pyridine ring and the replacement of the pyridine ring by quinoline resulted in complete loss of antitumour activity.

Most of the complexes that were investigated showed significant (T/C, % $\geq$ 125) antitumour activity, the highest level of activity was, however, exhibited by Cu(PCPH)Cl_2 complex (T/C, % = 180% at 60 mg/kg dosage). Since Cu(PCPH) Cl_2 was one of the most active agent of the series in the P 388 lymphocytic leukemia test system, it was also tested against a variety of other trans-

planted tumour systems, i.e., Sarcoma 180 (T/C, % = 195 at 40 mg/kg/day), Ehrlich carcinoma (T/C, % = 207 at 60 mg/kg/day) and leukemia L 1210 (T/C, % = 206 at 60 mg/kg/day). Compound Cu(PCPH) Cl_2 showed activity in each of these test systems indicating that the agent possessed a wide spectrum of antitumour activity.

Essentially complete inhibition of the incorporation of of thymidine - ^3H into DNA of Sarcoma - 180 ascites cells occured when the radioactive precursor was injected intraperitoneally into mice bearing 6-day accumulations of neoplastic cells 15 min. after the administration of 1-formylisoquinoline thiosemicarbazone, IQ-1, at a dose level of 25 mg/kg and was allowed 1 hr to be utilized (6). This degree of blockade persisted for upto 12 hr after IQ-1, but by 24 hr after the chelating agent, inhibition was completely relieved. The data (Table) indicate that at the same level of dosage Cu(PCPH) Cl_2 caused essentially complete inhibition of thymidine - ^3H into DNA at 6 hr. Twelve hours after, however, the inhibition by Cu(PCPH) Cl_2 was decreased, but by 24 hr after inhibition was completely terminated. Like α-(N) -heterocyclic thiosemicarbazones, the inhibition of the biosynthesis of RNA and protein was also produced by Cu(PCPH) Cl_2 however, these metabolic processes were considerably less sensitive than was the replication of DNA (6). Maximum inhibition of RNA synthesis was produced by IQ-1 3 hr after administration of the drug, and the level of the inhibition decreased slowly thereafter (6). Under essentially the same conditions, Cu(PCPH) Cl_2 inhibited the RNA synthesis at the same degree at 12 hr after administration of the drug, but by 18 hr after inhibition by Cu(PCPH)Cl_2 was decreased. Thus, the degree of inhibition of DNA synthesis of Sarcoma 180 cells *in vivo* was very comparable to that observed for α- (N)-heterocyclic carboxaldehyde thiosemicarbazones. These agents have long been recognized as potent cytotoxic agents which exhibit tumour-inhibitory actions that have been attributed to their abilities to function as tridentate chelators of transition metal ions (7,8). Early structure-activity studies established that the conjugate N*-S* tridentate ligand system was essential feature for compounds to exhibit antitumour activity (7). The above results, however, indicated that the ligands that are similarly tridentate but which binds metal through an N*-N*-N* array of donor atoms are also potent inhibitors of cell growth and that, as in the α- (N)-heterocyclic carboxaldehyde thiosemicarbazones, complexation to copper increases tumour inhibitory activity (9).

Table 1 : Incorporation of Thymidine - ^{3}H, Uridine - ^{3}H and DL-Leucine - ^{14}C into DNA, RNA and Protein, respectively, of Sarcoma 180 Ascites Cells Treated with Cu(PCPH) Cl_2.

Dose mg/kg	Protreatment (hr) before radioactive precursor	% inhibition		
		Thymidine - ^{3}H into DNA	Uridine - ^{3}H into RNA	DL-Leucine ^{14}C into Protein
36.2	6	96		
	12	70	44	28
	18	18	15	
	24	0		

Mice bearing 6-day implants of Sarcoma 180 ascite cells received a single intraperitoneal injection of Cu(PCPH)Cl_2 per kilogram of body weight. At the indicated times, thereafter, animals received the appropriate radioactive tracer by ip injection, which was allowed 1 hr to be incorporated. Control specific activities were 22.2 cpm/n mol 11.7 cpm/n mol and 6520 cpm/mg for thymidine - ^{3}H, Uridine - ^{3}H and Leucine - ^{14}C incorporation, respectively. These values reprosent the mean (± standard error) or results obtained with 8-16 mice analyzed separately.

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METAL IONS COMPLEXATION PROPERTIES OF ANTITUMOR ANTHRACYCLINES. DIFFERENT CYTO AND CARDIOTOXICITY OF COPPER(II) CHELATES

F. Morazzoni, L. Pellicciari-Bollini, F. Piccinini, E. Monti, L. Paracchini and R. Supino

INTRODUCTION

The anthracycline antitumour drugs are thought to display their therapeutic action through cytotoxic radicals. The enzymatic generation of carbon centered radicals, possibly followed by an electron transfer from the semiquinone radical to the molecular oxygen, has been suggested as a step in drug induced cell damage(1). Very recently an alternative mechanism of "non enzymatic" O_2 reduction was proposed, the coordination of anthracycline to a bivalent copper center having been indicated as an essential step to activate the drug(2).

In spite of the possible involvement of copper in the action mechanism of the antitumour drugs, a detailed physico-chemical characterization of the Cu(II)/ anthracycline derivatives was lacking.

We attempted this investigation with the aim of demonstrating that the structural properties of complexes can account for their antitumour action. At the same time structure-function relationships have been considered a reason for drug cardiotoxicity.

MATERIALS AND METHODS

Aqueous solutions of anthracyclines(Doxorubicin, 4'-Epi Doxorubicin) 10^{-3}M and $CuCl_2$ 10^{-3}M were mixed in different drug/Cu(II) molar ratios (r) and the pH value adjusted as desired by addition of HCl or NaOH (10^{-2}M). E.s.r. spectra were recorded on a Varian E-109 spectro-

meter, equipped with an automatic temperature control. The X-band spectra were recorded at -150 °C and the g values standardized by DPPH.

The cardiotoxic effects of the different drugs at a concentration of 50 μg/ml were tested on spontaneously beating atria isolated from female Sprague Dawley rats and incubated in Tyrode solution(NaCl 138 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 12.8 mM, NaH_2PO_4 0.48 mM, glucose 6.94 mM; pH 7.2). The isometric contractile force was recorded as dF/dt for 60'. Differences in inotropic effects were evaluated by means of the analysis of variance(3).

Cytotoxicity of the compounds was evaluated on HeLa cells. After treatment with different drugs at different concentrations for 60', cells were incubated in a drug-free culture medium(Eagle Basal Medium) for 72 h. The number of viable cells was than evaluated by dye exclusion.

RESULTS

Physico-chemical characterization of the Cu(II)/anthracycline derivatives

Different behaviours were observed depending both on the drug and on (r) values. The amino-sugar steric configuration and the drug/Cu(II) molar ratio (r) were discriminating factors in obtaining different copper complexes.

1/1 Drug/Cu(II) molar ratio. The addition of $CuCl_2$ to a Doxorubicin solution resulted in the formation of a square planar Cu(II) derivative where each drug molecule is coordinated to two metal centers. Chelating moieties are the hydroxo-antraquinone groups at C_{11}, C_{12} and C_5, C_6. The major structural evidence comes from the e.s.r. investigation, which allows the distinguishing of the tetragonal symmetry of CuO_4 chromophore and reveals spin coupling interaction between the paramagnetic metal centers. 4'-Epi Doxorubicin showed an identical chromophore structure, provided that the coordination involves only the hydroxo-antraquinone moiety at C_{11},C_{12}. The hydrogen bond between

the chelating groups at C_5,C_6 and the OH at C_4' prevents further chelation to copper. Fig.1 shows the coordinating schemes for the two classes of derivatives and each structure is associated to the corresponding e.s.r. spectrum (4,5,6).

Cu(II) derivatives of the two anthracyclines are very similar in the chromophore structure, but they have different behaviour towards oxygen. The formation of 4'-Epi Doxorubicin derivative is much more improved by the presence of O_2 in solution than is the doxorubicin derivative. This leads to the conclusion that, though no reduced paramagnetic oxygen forms are detectable in the experiments, O_2 enters the Cu(II) coordination field and withdraws metal electrons, an effect dependent on the electronic π density at the metal and on the ability of anthracycline to accept the Cu electrons. The polynuclear derivative(Doxorubicin) has π^* drug orbitals delocalized along the Cu-Drug chain and is more suited to accept the d_π copper electrons than is the mononuclear (4'-Epi Doxorubicin). These results are consistent with the data of oxygen consumption of Cu(II)/ Doxorubicin published by Wallace (2) and suggest that the strength of the Cu-O_2 bond depends on the competition for the d_π Cu electrons between the π^* O_2 orbitals and the π^* drug orbitals.

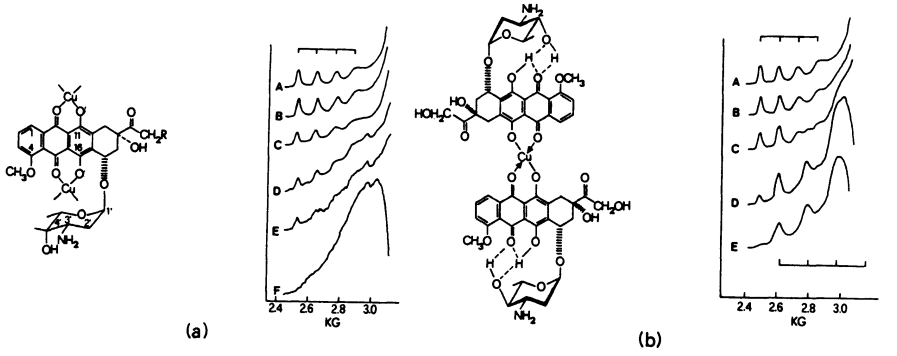

Fig.1 a) Molecular scheme of the Cu(II)/Doxorubicin derivative and its e.s.r. spectrum. The different lines from A to E are for different pH values: 5, 5.5, 6, 6.5, 7 b) ibidem for Cu(II)/Epidoxorubicin

n/1 Drug/Cu(II) molar ratio (n=5,10). The drug excess induces the formation of a new Cu(II) derivative,which is the same for both the considered drugs. Anthracycline behaves as"stacked" ligand and the chromophore structure is consistent with a decrease of charge on the metal center. The e.s.r. investigation suggests a mononuclear Cu complex (Fig.2). The lower electron donor power of the anthracycline in the"stacked" configuration explains why the formation of the new complex is independent of the O_2 presence, confirming the essentially π nature of the Cu-O_2 interaction.

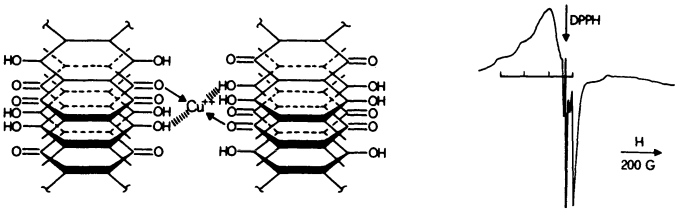

Fig.2 Molecular scheme of the Cu(II)/Doxorubicin(4'-Epi Doxorubicin) derivative when (r) = 10, and its e.s.r. spectrum at pH 6.5

Drug interaction with Cu-Zn Superoxide Dismutase

As a consequence of the revealed interaction between Cu(II) and O_2, in the presence of anthracycline, it becomes important to verify whether interaction can occur between the drug and the antioxidant enzyme containing bivalent copper. The e.s.r. investigation reveals that the interaction of anthracyclines with Cu-Zn Superoxide Dismutase is not dissimilar from that observed when the drug is in a 10:1 molar ratio to $CuCl_2$. Each copper is able to bind several anthracycline molecules.

Antitumour effect and cardiotoxicity

The preliminary results were obtained by using Cu(II)/Doxorubicin in a 2:1 Drug:Cu(II) molar ratio. The structure of the derivative at (r) = 2 is similar to that at (r) = 1. The choice of (r) = 2 was to

avoid precipitation of the more insoluble derivative at (r) = 1.
A 60' treatment gave an antitumour effect, measured as ID_{50}, no different from that of the free drug (ID_{50} Doxorubicin = 300 ng/ml; ID_{50} Cu/Doxorubicin = 360 ng/ml). On the contrary, a significant difference was observed between the cardiotoxic effect of Doxorubicin and of its Cu(II) derivative as measured after 60' perfusion (Fig.3).

Though definitive conclusions cannot be drawn at the present, our suggestion is that the presence of the metal inhibits the effects of cardiotoxic radicals. The reasons could be:
lack of radical formation due to the inhibition of the electron transfer from the anthracycline to O_2 in the presence of Cu(II);
fast reaction of the formed toxic radicals with Cu(II) centers.

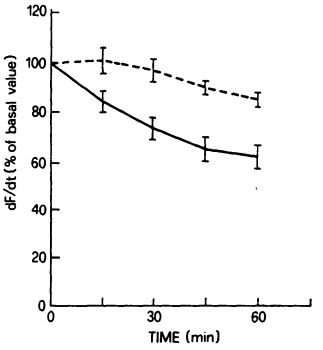

Fig.3 Time course of contractile force for _____Doxorubicin
_ _ _ _ Cu(II)/Doxorubicin (mean ± S.E. of 6-8 preparations)

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CYTOSTATIC AND ANTITUMOUR PROPERTIES FOR A NEW SERIES OF Pt(II) COMPLEXES WITH CYCLOPENTYLAMINE

V. Scarcia, A. Furlani, A. Papaioannou, D.G. Craciunescu and A. Doadrio

INTRODUCTION

It is well-known that the alicyclic amine derivatives of cisplatin are less toxic as the ring size increases from cyclopropylamine to cyclohexylamine, whereas the antitumour activity remains largely unalterated (1). Nevertheless the cyclopentylamine complex shows a larger spectrum of activity than the cyclohexylamine analog.

On the basis of these observations, we thought it to be of interest to extend our researches on Pt(II) complexes with cyclopentylamine (cpa) having as bidentate leaving ligands phthalates (PA) and isophthalates (IPA) derivatives, since the dicarboxylate anion ligands appear to be very interesting (2, 3).

This communication reports the in vitro cytostatic and the in vivo antileukaemic activity of the new complexes.

MATERIALS AND METHODS

The in vitro cytostatic activity was tested on KB cells, an established human tumour cell line, according to Protocols of National Cancer Institute (Bethesda, MD) (4).

The in vivo antitumour activity was performed against mice bearing the established P388 and L1210 leukaemias with the standard protocols (4).

RESULTS AND DISCUSSION

The results of the in vitro assay are summarized in Table 1. Five complexes showed interesting cytostatic properties with ID_{50} values lower than 1 μg/ml. In some cases these values were comparable to that of cisplatin, determined by us in the same experimental conditions.

The Table 2 summarized the best results obtained with our complexes against P388 and L1210 leukaemias.

Table 1. Cytostatic activity against KB cell growth.

Compounds*	ID_{50} values**
cisplatin	0.11 (3.6×10^{-7} M)
$Pt(cpa)_2$(3-nitro-PA)	5.74 (1.0×10^{-5} M) °
$Pt(cpa)_2$(4-nitro-PA)	0.32 (5.6×10^{-7} M) °
$Pt(cpa)_2$(5-nitro-PA)	0.99 (1.7×10^{6} M)
$Pt(cpa)_2$(3,4-dinitro-PA)	>10.00
$Pt(cpa)_2$(3,5-dinitro-PA)	>10.00 °
$Pt(cpa)_2$(3,6-dinitro-PA)	>10.00 °
$Pt(cpa)_2$(IPA)	0.38 (7.1×10^{-7} M) °
$Pt(cpa)_2$(2,4-dinitro-IPA)	>10.00
$Pt(cpa)_2$(2,5-dinitro-IPA)	0.84 (1.3×10^{-6} M) °
$Pt(cpa)_2$(5-sulfo-IPA)	0.29 (4.7×10^{-7} M)

* The compounds were previously dissolved or suspended (°) in 5 % dextrose in water.

** The results are expressed as concentrations (g/ml MEM) for which the cells showed a 50 % growth inhibition (ID_{50}).
In parentheses the ID_{50} values are reported as molarity.

Against the P388 leukaemia all complexes exhibited good antitumour activity, comparable and higher than cisplatin. Whereas the L1210 tumour system appeared to be more resistant.

The highest antitumour effectiveness seemed to be corre

lated to the presence of the 5-sulfo-IPA, IPA and nitro-PA derivatives as leaving ligands. In fact these complexes mantained an interesting activity also against L1210 system.

The interest toward these last compounds, with respect to cisplatin, is emphasized when we compare their antileukaemic activity with their LD_{50} values (Table 2).

The high in vivo effectiveness of almost all complexes is not associated with a high in vitro cytostatic activity, in fact only the most effective in vitro compounds show a significant cell growth inhibition. This finding should be ascribed to a particular unreactivity of platinum carboxylate complexes, which has been already emphasized by other researchers, who have postulated the involvement of an in vivo activation mechanism (2).

Table 2. Antitumour activity against P388 and L1210 leukaemias.

Compounds*	Dose (mg/Kg)	P388 (T/C %)**	L1210 (T/C %)**	LD_{50} (mg/Kg)
cisplatin	5	230	189	13
$Pt(cpa)_2$(3-nitro-PA)	50	290	198	400
$Pt(cpa)_2$(4-nitro-PA)	50	270	160	500
$Pt(cpa)_2$(5-nitro-PA)	100	295	178	580
$Pt(cpa)_2$(3,4-dinitro-PA)	100	247	136	800
$Pt(cpa)_2$(3,5-dinitro-PA)	100	235	135	625
$Pt(cpa)_2$(3,6-dinitro-PA)	50	252	150	550
$Pt(cpa)_2$(IPA)	50	291	187	550
$Pt(cpa)_2$(2,4-dinitro-IPA)	50	167	117	610
$Pt(cpa)_2$(2,5-dinitro-IPA)	50	198	137	650
$Pt(cpa)_2$(5-sulfo-IPA)	50	310	250	150

* The compounds were administered by i.p. injections on days 1, 5, 9 as suspensions in 5 % dextrose in water.

** The antitumour activity is expressed as mean survival time of treated mice x 100 / the mean survival time of the controls.

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1-METHYLCYTOSIN-COMPLEXES OF Pt(IV)

F. Schwarz, H. Schöllhorn, U. Thewalt and B. Lippert

INTRODUCTION

Pt(IV)-compounds of type cis,cis,trans-$PtL_2Cl_2(OH)_2$ or cis-$Pt(NH_3)_2Cl_4$ are frequently less toxic and better soluble than cis-PtL_2Cl_2 or cis-$Pt(NH_3)_2Cl_2$. It is not clear whether these Pt(IV)-compounds are intracellularly reduced to Pt(II)-compounds before they react with DNA /1, 2, 3, 4/ or if they react directly with DNA /5, 6/.

As has recently been demonstrated /7, 8/, trans,trans,trans-$[Pt(NH_3)_2(OH)_2(1\text{-MeC-}N^3)_2](NO_3)_2$ (1-MeC = model nucleobase 1-methylcytosine) (I) undergoes (N^3,N^4)-chelate formation (II) to form finally the most thermodynamically stable product trans,trans,trans-$[Pt(NH_3)_2(OH)_2(1\text{-MeC-}N^4)_2](NO_3)_2$ (III). In III the 1-MeC-ligand is present as the rare iminooxo tautomer form, stabilized through Pt-binding via N^4.

It was the aim of this study to prepare other Pt(IV)-complexes of 1-methylcytosine that might show metal migration from N^3 to N^4. For this purpose cis,trans-$[Pt(NH_3)_2(OH)_2(1\text{-MeC-}N^3)Cl]Cl\cdot 2H_2O$, 1, was prepared.

RESULTS

Molecular Structure of Cation 1. As shown in figure 1, Cl^- and 1-MeC remain in cis-position after oxidation of

the educt cis-[Pt(NH_3)$_2$(1-MeC)Cl]Cl·H_2O with H_2O_2. Although NH_3- and OH-groups can not be distinguished unambiguously by x-ray crystallography, the temperature factors of N- and O-atoms and the hydrogen bonding pattern in the crystal lattice favour the assignment of NH_3- and OH-groups. This interpretation is further supported by the fact, that a permutation of N- and O-atoms leads to higher R-factors.

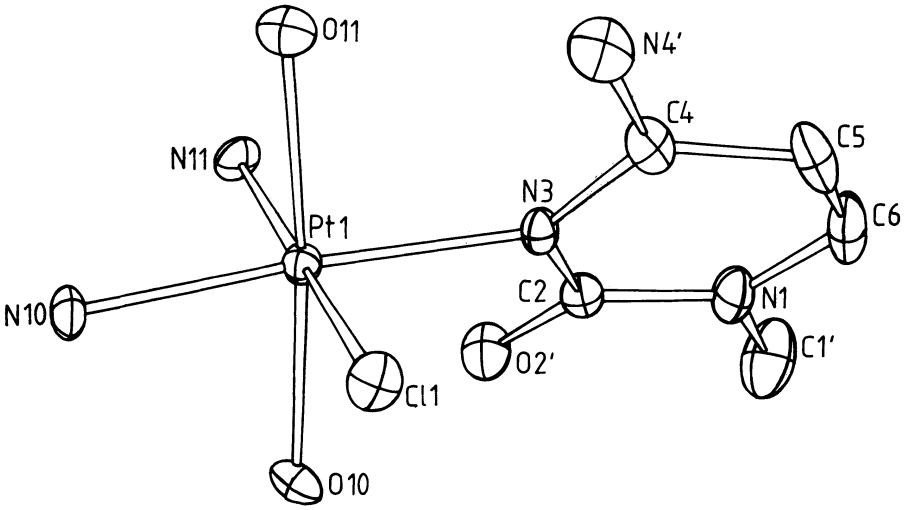


Fig. 1 Crystal structure of 1

The coordination sphere of platinum is roughly octahedral. The bond distance Pt-N^3(cyto) of 2,090(5) Å is comparable with corresponding distances in Pt(II)-1-MeC-compounds /9, 10/.
The cytosine ligand is planar. With the N10, N11, N3, Cl1-plane it forms an angle of 55°. This dihedral angle is small compared to those of corresponding Pt(II)-

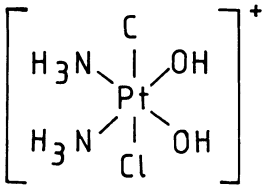
complexes /5/ and a result of the steric interactions of the exocyclic groups of the cytosine ligand (NH_2(4),O(2)) and the axial OH-groups.
The essential structural data of the cation of 1 are summarized in Table 1:

Tab. 1 Distances [Å] and Angles (deg.) in 1

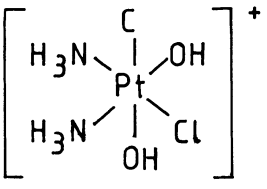
Pt1 - Cl1	2,324(2)	Pt1 - O10	1,985(5)
Pt1 - N10	2,040(5)	Pt1 - O11	2,027(5)
Pt1 - N11	2,023(5)	Pt1 - N3	2,090(5)

Cl1 - Pt1 - N10	87,7(2)	N11 - Pt1 - O10	92,2(2)
Cl1 - Pt1 - N11	177,9(2)	N11 - Pt1 - O11	86,6(2)
Cl1 - Pt1 - O10	89,4(2)	N11 - Pt1 - N3	89,4(2)
Cl1 - Pt1 - O11	91,7(1)	O10 - Pt1 - O11	174,7(2)
Cl1 - Pt1 - N3	91,9(2)	O10 - Pt1 - N3	92,1(2)
N10 - Pt1 - N11	91,1(2)	O11 - Pt1 - N3	93,0(2)
N10 - Pt1 - O10	83,6(2)	Pt1 - N3 - C2	116,9(4)
N10 - Pt1 - O11	91,2(2)	Pt1 - N3 - C4	124,4(5)
N10 - Pt1 - N3	175,8(2)		

Solution behavior of cis,trans-[Pt(NH_3)$_2$(OH)$_2$(1-MeC)Cl]Cl·2H_2O ,1. Conditions, which led to chelate formation in the case of trans,trans,trans-[Pt(NH_3)$_2$(OH)$_2$(1-MeC)$_2$](NO_3)$_2$ (I), did not afford a chelate with 1. Rather, partial decomposition (formation of free ligand) and formation of a new complex 2 was observed. 2 is tentatively assigned to either of the following two isomers,



or



based on results obtained from several reactions of 2

with HCl and the reductand $H_2C_2O_4$. 1 and 2 differ slightly in their vibrational spectra (e.g. Raman bands at 530 s, 557 s, 586 s in 1 and 545 s, 582 vs in 2) and their ^{1}H-NMR-spectra (H^5: 5,86 ppm (1) and 5,90 ppm (2)).

EXPERIMENTAL

Preparation of 1. 400 mg of cis-[Pt(NH_3)$_2$(1-MeC)Cl]Cl were dissolved in 10 ml H_2O at room temperature. 7 ml H_2O_2 (30%) were added in small portions, which caused a temporary rise in temperature to 60° C. Then the solution became yellow and a strong evolution of gas was observed. Slow evaporation at 22° C gave 246 mg (55%) of 1 as yellow crystals.

Analysis for $PtC_5H_{19}N_5O_5Cl_2$:

found:	C 12,35%	H 3,58%	N 14,08%	Cl 14,51%
calcd.:	C 12,12%	H 3,87%	N 14,14%	Cl 14,32%

Preparation of 2. 115 mg cis,trans-[Pt(NH_3)$_2$(OH)$_2$(1-MeC)Cl]Cl·2H_2O were dissolved in 2 ml H_2O, kept at 70° C for 5 hours and cooled to room temperature. 27 mg (23%) of deep yellow crystals were isolated on slow evaporation.

Analysis for $PtC_5H_{17}N_5O_4Cl_2$:

found:	C 12,72%	H 3,39%	N 14,79%	Cl 15,02%
calcd.:	C 12,58%	H 3,59%	N 14,68%	Cl 14,86%

Crystallography

X-ray measurements were carried out on a PHILIPS PW-1100 diffractometer at room temperature with graphite monochromator Mo Kα radiation (λ=0.71069 Å). The compound crystallizes in the triclinic space group $P\bar{1}$ with a=11.003(4), b=9.430(s), c=7.445(3)Å, α=107.49(4), β=92.53(4), γ=101.81(5)°, V=716.6Å^3, Z=2. Experimental details were as follows: 2359 unique reflections, 2348 reflections used in calculations

($I>\sigma I$), $\Theta/2\Theta$scan ($\Theta_{max}=25°$), $\mu=97.7cm^{-1}$, anisotropic temperature factors, $R=0.032$, $R_w=0.035$ ($w^{-1}=\sigma^2(F) + 0.002\ F^2$), hydrogen atoms not localized.

SUMMARY

A 1-methylcytosine complex of Pt(IV) has been prepared, structurally characterized and its solution behaviour studied. N^3,N^4-chelate formation was not observed. Studies are in progress to elucidate the possible role of Cl^- in preventing chelate formation.

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EFFECTS OF NICKEL-LAPACHOL IN HYPOXIC CELLS

K.A. Skov, H. Adomat and N.P. Farrell

ABSTRACT

Previous studies on the radiosensitizing properties of the lapachol chelate of nickel suggested a possible chemosensitizing role for this complex. Hypoxic mammalian cells become more sensitive to the toxic action of cisplatin when also treated with nickel-lapachol. There is less sensitizing effect in aerobic cells; lapachol alone shows less effect than its nickel complex. It is suggested that the interaction occurs at the DNA level.

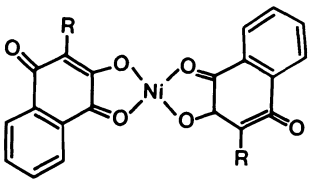

Figure 1: Structure of Nickel-Lapachol (NiL_2), R=isoprenyl

INTRODUCTION

Hypoxia and Radiosensitizers - Because of rapid growth and distance from the capillaries, tumour cells may outstrip the oxygen supply, and become acutely or chronically hypoxic. Hypoxic cells in tumours were reported to be resistant to radiotherapy some 30 years ago (1), and may also be refractory to certain chemotherapeutic regimes. One of the many approaches taken by the radiobiologist is the use of radiosensitizers to combat the so called hypoxic problem. Nitroimidazoles, the most studied class of radiosensitizing compounds, are proposed to act by a mechanism dependant on their redox properties (2). Electron abstraction from the irradiated

target (DNA) enhances the damage in a manner similar to oxygen itself (additional breaks and other damage (3)). This type of compound, as well as radiosensitizing hypoxic cells (2,4) also causes DNA damage (3,6) and is toxic to hypoxic cells (4), properties again related to redox properties (5). Unfortunately, accompanying toxic side-effects of these compounds in the clinic has prevented attaining maximum radiosensitizing, leading to a search for other electron affinic radiosensitizing compounds. We are investigating metal complexes in this regard.

Lapachol chelates - Lapachol, a 1,4-naphthoquinone isolated from tropical trees 130 years ago, shows anti-tumour activity (7). In clinical trials, it was found that plasma levels high enough to affect tumours in patients could not be attained (8) and more soluble analogues have been synthesized.

The possibility that some transition metal complexes of lapachol might have radiosensitizing properties was proposed because of their properties as chemiluminescent catalysts (Farrell, unpublished). The radiosensitizing properties of some complexes have been examined, were presented recently (9) and may be summarized as follows:

(a) of the series examined, the nickel complex, $[Ni(lapachol)_2]$, NiL_2, was the best radiosensitizer of hypoxic CHO cells;

(b) this radiosensitization may be partially due to enhancement of radiation induced breaks in DNA (like electron affinic compounds);

(c) when hypoxic cells are incubated with NiL_2 (no radiation) breaks in DNA are produced, which are not seen in oxic cells;

(d) the toxicity of NiL_2 is somewhat greater towards hypoxic than aerobic cells.

Further details on radiosensitizing studies will be published elsewhere.

Rationale - The toxic properties (c, d above) which may partially explain the interaction with radiation may also enhance cytotoxicity of other agents which do not normally show selectivity for hypoxic cells. Our first study of this nature shows that NiL_2 enhances the toxicity of cisplatin in hypoxic and aerobic mammalian cells.

MATERIALS AND METHODS

Toxicity in CHO cells was assessed according to (4). Briefly, stirred suspensions of complex(es) in α-medium with 10% fetal calf serum (GIBCO) were rendered hypoxic by nitrogen flow for 45 minutes before addition of a small aliquot of cells (final conc $2x10^5$ cell/ml). Cells were removed at given times during the 37°C incubation, washed twice, and plated in α-medium; colony forming ability was assessed after 7 days.

Breaks in DNA of cells exposed to a given agent are assessed using the unwinding technique (10) with ^3H and ^{14}C thymidine label incoporated into DNA.

Cisplatin was a gift of David Bull Laboratories, Mulgrave, Australia; the lapachol for the synthesis of the complexes (Farrell, unpublished) was provided by his chemistry colleagues at the Universidade Federal de Minas Gerais, Brazil. Propylene glycol (final conc 1%) was used to facilitate solution.

RESULTS AND DISCUSSION

Toxicity of NiL_2 - The chelate is moderately toxic in CHO cells, with plating efficiencies (PE) typically 0.3 and 0.43 in hypoxia and air respectively after one hour incubation at 37°C with 400μM NiL_2 (control PE 0.7). This toxicity has been measured at other concentrations and for longer times. Thus while NiL_2 toxicity is not greatly affected by the absence of oxygen, many more breaks in DNA are detected after hypoxic incubation with NiL_2 than in oxic cells (Figure 2).

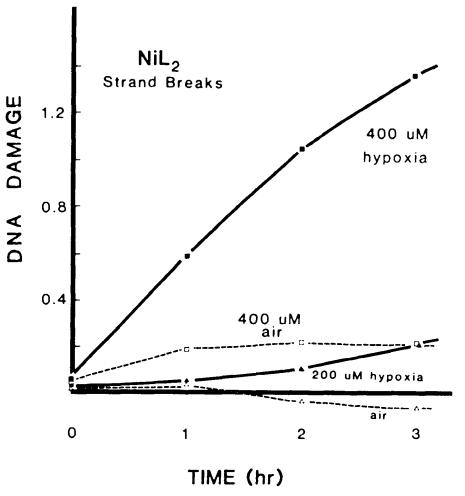

Figure 2:
Breaks in DNA are produced in cells incubated under hypoxic conditions with 400 μM NiL_2. At 800 μM (2L), lapachol alone produced the same degree of damage as 200 μM NiL_2. (Breaks are assayed using the unwinding technique, with double label .)

Toxicity of Lapachol - The ligand itself is less toxic than NiL_2. Some DNA damage can be detected in cells after hypoxic incubation with lapachol, but far less than NiL_2.

Toxicity of cisplatin - Our results in CHO cells agree with those of others (PE _ 0.05 after 1 hr with 10μm cisplatin). In this laboratory, there is no difference between the toxicities in the presence or absence of oxygen. This has been reported by others in V79 cells (11 and R. Durand, pers. comm. 1987); however, higher toxicity in hypoxia has also been reported (12).

NiL_2 and Cisplatin - There is a marked increase in the toxicity of cisplatin in cells when they are also treated with NiL_2. In air, the effect of NiL_2 on cisplatin toxicity is approximately the same as the ligand alone. This chemosensitizing effect may be higher in hypoxia for lapachol alone, but the presence of the Nickel center produces a considerable increase in toxicity. The effect of lapachol chelates of other metals will be measured. These preliminary results appear greater than additive; however, further experiments and rigorous analysis (eg. 14) will be required to confirm synergy.

TOXICITY: APPROXIMATE PLATING EFFICIENCY AT 2 HOURS

		-	+ cisplatin (5 μM)
Control		0.7	0.01
Lapachol (800 μM)	N_2	0.4	0.002
	O_2	0.5	0.004
NiL_2 (400 μM)	N_2	0.06	0.00015
	O_2	0.20	0.006

Possible Mechanisms - During the course of our radiosensitization studies on NiL_2, we have measured thiol levels in cells after exposure to NiL_2 and found no depletion. NiL_2 itself does not appear to bind to DNA as assessed by inhibition of restriction endonucleases (14), whereas cisplatin binding to DNA has

been studied by a variety of techniques including (14). Nickel ions have been shown to have cross- linking ability (15,16), and preliminary results indicate that the chelate NiL_2 may also form DNA-protein crosslinks in hypoxia using the alkaline elution technique. The breaks which are formed in hypoxia are rapidly repaired (similar to x-ray repair kinetics) (Adomat, unpublished).

It is possible that these breaks interact by facilitating formation of more cisplatin adducts, or by making repair more difficult. Studies are in progress to determine the effect of cisplatin on repair of NiL_2 breaks, and NiL_2 on incidence and repair of cisplatin crosslinks. Other chemotherapeutic agents which bind to DNA will also be assessed in the presence of NiL_2.

Chemosensitization - There has been considerable interest in the use of nitroimidazoles to enhance the action of chemotherapeutic agents, particularly in the resistant, hypoxic fraction of tumours (eg. 17, 18). The chemosensitizing action of these compounds is related to their toxicity, i.e. reduction in hypoxia, and thiol depletion. As stated above, both toxicity and radiosensitization are related to redox properties. To date, nitroheterocycles are the main class of compounds studied which have these three actions: radiosensitization, toxicity, and chemosensitization of hypoxic cells.

It is suggested that we have identified a new class of compounds with properties similar to nitroimidazoles. The redox properties of these compounds will be investigated. The nickel-lapachol complex, as described here, has certain properties in hypoxia which make it and its analogues attractive for further investigation in conjunction with radiation, with cisplatin, and with other chemotherapeutic agents, both in vitro and in vivo.

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PLATINUM(II) COMPLEXES WITH AMIDES OF PYRIDINECARBONIC ACIDS AND SOME DIAMIDES

A.I. Stetsenko, L.S. Tikhonova and L.I. Iozep

The mechanism of antitumour action of cis-DDP suggests reactions of this complex with bases of DNA [1]. It is also supposed that Pt(II) complexes influence the interaction DNA-protein and are fixed by blood and tissue proteins [2]. The separation and identification of Pt(II) complexes is a complicated process. Therefore it is reasonable to simulate this interaction using simpler ligands.

Pt(II) complexes with amides of isomeric pyridinecarbonic acids - nicotinamide(NA), isonicotinamide(INA) and amide of pycolinic acid(APA) have been investigated. Non-electrolytic isomeric complexes $[PtL_2X_2]$, (L - amide X - Cl^-, Br^-, I^-), cation complexes of tri- and tetramine types - cis-$[Pt(NH_3)_2LCl]Cl$ and isomeric $[Pt(NH_3)_2L_2]Cl_2$ have been isolated [3,4]. The composition and coordination formulas of compounds have been proved by elementary analysis, conductometric measurements and longwave IR spectroscopy. The purity of compounds has been confirmed by the method of thin-layer chromatography.

In the IR spectra of this compounds the frequencies of stretching vibrations of the $CONH_2$-group and in the ring are increased compared with free ligands, which points to the absence of bond between the amide-group and platinum. In the ^{1}H-NMR spectra of trans-$[PtL_2X_2]$ in DMSO-d_6 for protons H(2)-H(6) we observe greater downfield shifts, which are located adjacent to the he-

terocyclic nitrogen atom. On this basis it is inferred that monodentate coordination of ligands is effected through the heterocyclic nitrogen atom.

The constants of acidic dissociation of cis-$[PtL_2(H_2O)_2]^{2+}$ where L - NA, INA, have been estimated. The 17-18-fold enhancement of acidic properties of coordinated water molecules on substitution of a NH_3-molecule by NA or INA is accounted for by the π-acceptor properties of amide ligands [5].

The polarographic reduction of cis- $[Pt(NH_3)_2LCl]Cl$ on DME in 0.1 M Na_2SO_4 has been studied. The characteristics of polarographic waves of the triamines investigated are given in table 1.

Table 1. Characteristics of polarographic waves of reduction on DME of cis-$[Pt(NH_3)_2LCl]Cl$(1.10^{-3}M in 0.1M Na_2SO_4)

L	$E_{1/2}$, V	I_d, μA	Δ lgI/Δ lgH	E/Δ lgI/I_d-I, V
INA	-0.41	5.08	0.74	0.18
NA	-0.42	5.10	0.42	0.22
APA	-0.86	4.48	0.63	0.25
NH_3	-0.89	7.12		

It is evident from Table 1 that reduction of all complexes is an irreversible process. The coulonometric measurements have proved the twoelectron nature of the reduction process.

Comparison of $E_{1/2}$ for the compounds concerned shows that the substitution of NH_3 by NA and INA leads to an enhancement of the reducing ability of the complex. This effect is attributed to the π-acceptor ability of amides of pyridinecarbonic acids which brings about an increase in effective positive charge of the central atom and stabilization of the lowest free mole-

cular orbital. It is suggested that electrons are transferred to the lowest free molecular orbital resulting from the overlapping of d-orbital of Pt(II) and π-antibonding orbital of the amide group.

The oxidation of Pt(II) in cis-triamines $[Pt(NH_3)_2LCl]Cl$ has been investigated by the of cyclic voltammetry on a platinum electrode in solution 1M HCl(Table 2).

Table 2. Characteristics of cyclic voltammetry curves on a platinum electrode of cis-$[Pt(NH_3)_2LCl]Cl$(1.10^{-3}M in 0.1M NaCl+ 1M $HClO_4$)

Ligand	$_aE_p$, V	$_cE_p$, V	ΔE_p, V	$E_{1/2}$, V
INA	0.73	0.46	0.27	0.80
NA	0.72	0.44	0.28	0.78
APA	0.68	0.42	0.22	0.65
NH_3	0.62	0.40	0.22	0.65

The value of $E_{1/2}$ was obtained upon oxidation of complexes on a rotating platinum electrode(2000 r.p.m.).

Oxidation is to be considered as a quasireversible process, which follows from the value of ΔE_p and the dependence of the relationship $_aI_p/_cI_p$ on the rate of the sweep. The comparison of $_aE_p$ values shows that the substitution of NH_3 by amides of pyridinecarbonic acids hinders to some extent the oxidation process. This may be due to the stabilization of the highest occupied molecular orbital resulting from the π-acceptor properties of the ligand. The coulonometric measurements carried out at the potential +0.8 V have shown that the oxidation of cis-$[Pt(NH_3)_2INACl]Cl$ is a twoelectronic process irrespective of the nature of the supporting electrolyte. In contrast to this, reduction of the complex in 1M $HClO_4$ is a stepwise process. Coulonometric measurements have shown a monoelectronic transfer on

each stage, which is evidence of the stepwise nature of the platinum reduction: Pt(IV) → Pt(III) → Pt(II).

Cation amide platinum blues with amide anions - (L-H) and platinum proportions Pt: NH_3:(L-H) = 1:2:1 have been separated in reactions between the product of hydrolysis of cis-DDP with INA, biuret, malondiamide. In order to estimate the oxidation state of platinum, use is made of cerimetric titration, cyclic voltammetry and XPS. The complex with biuret contains Pt(II), which is inferred from the results of cerimetric titration(two electrons) and the proximity of the potentials of anode and cathode peaks to respective peaks of Pt(II) complexes. According to cerimetric data the oxidation state of platinum in blues with INA and malondiamide is above two: in blues with INA 2.5+ and with malondiamide about 3+. Oxidation of blues proceeds at a more positive potential than in case of Pt(II) complexes. The coulonometric measurements carried out at the potential of anode peak corresponds to the oxidation state 2.5+ in blues with INA and 2.8+ in blues with malondiamide. However, the ionization energy of platinum in blues $E_{Pt\ 4f7/2}$ equals 72.2 eV, which corresponds to the oxidation state of platinum 2+. The equivalence of platinum atoms in the compounds has been proved by XPS. It has been suggested that the reaction between cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and INA and malondiamide is atteded by partial oxidation of platinum and the formation of polynuclear compounds with bridged amide anions and a platinum-platinum bond.

METHODS

The IR spectra were recorded on "Specord-75IR" spectrometer and the ^{1}H-NMR spectra on "Tesla BS-487C, 80MHz". The polarographic measurements were performed using a PPT-1 polarograph. The pH- and conductometric measurements were performed using a pH-121 pH-meter

and CD-1 conductometer. CV-curves were taken from SWA-1 apparatus and platinum electrode, satured Ag/AgCl reference electrode whose used.

For coulonometric measurements use was made of a potentiostate P-5827 M and IP-12 integrator. The recording of XPS was performed on a HP-5950 A spectrometer.

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BIDENTATE HYDROXYCARBOXYLIC ACID PLATINUM(II) COMPLEXES WITH ANTITUMOR ACTIVITY

T. Totani, K. Aono, Y. Adachi, M. Komura, O. Shiratori and K. Sato

INTRODUCTION

In an attempt to find a new class of platinum antitumor agents with high antitumor activity, little renal toxicity and good solubility, we synthesized the title complexes. As previously reported, several glycolatoplatinum complexes were successfully obtained (1, 2) and, we now report the synthesis, characterization and antitumor activity of a series of non-ionic hydroxocarboxylatoplatinum-(II) complexes.

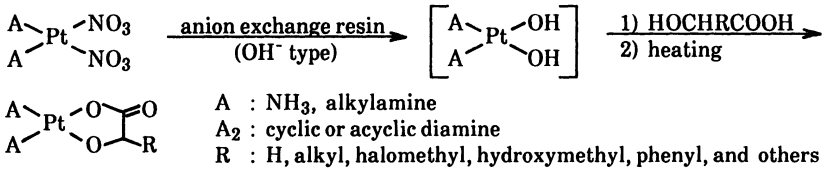
SYNTHESIS AND CHARACTERIZATION

Synthesis

These complexes, $[Pt(OCOCHRO)A_2]$, were obtained by adding an equimolar quantity of hydroxycarboxylic acid to $[Pt(OH)_2A_2]$ in water followed by heating. The synthetic route is shown in Scheme 1. Most complexes showed good solubility, i.e., >10 mg/ml in water, and were stable to recrystallization from water. Some complexes were recrystallized from a mixture of water-alcohol or water-acetone.

As a typical example of the synthetic method of the complexes formulated in $[Pt(OCOCHRO)A_2]$, that of the lactato complex is described here ($R = CH_3$, $A = NH_3$). A solution containing 1.5 g (4.25 mmol) of cis-diamminedinitratoplatinum(II) in 30 ml of water was passed through a column of 30 ml of anion exchange resin, Diaion SA10A (OH^- type). To the eluate was added 54 ml of an aqueous solution of freshly prepared DL-lactic acid (4.25 mmol) with stirring. The mixture was allowed to stand overnight, concentrated to 15 ml,

and heated at 65°C for 3 hr. The reaction mixture was further concentrated nearly to dryness. The remaining substance was purified by passing it through a column of silica gel (solvent; 70% EtOH) followed by recrystallization, which gave 0.63 g (yield 45%) of the objective complex.

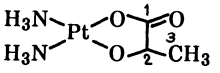


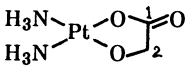
Scheme 1

Characterization of diammine(DL-lactato-O,O')Platinum(II)

This complex can be well characterized by SIMS and NMR spectroscopy in comparison with those in diammine(glycolato-O,O')platinum-(II) (2) as shown in Table 1. The data confirmed the bidentate (chelated) five-membered ring structure for the lactato ligand.

Table 1. Characterization data for diammine (DL-lactato-O,O')platinum (II).





254-S
(Ref. 2)

NMR (ppm, ^{195}Pt, ext. K_2PtCl_4 in D_2O, ^{13}C, int. dioxane)

$\delta_{C^2\text{-}H}$:	4.60 ($J_{^{195}Pt\text{-}H^2}$ = 28 Hz)	4.55 ($J_{^{195}Pt\text{-}H^2}$ = 34Hz)
$\delta_{^{13}C^2\text{-}H}$:	74.7 ($J_{^{195}Pt\text{-}^{13}C^2}$ = 15 Hz)	69.4 ($J_{^{195}Pt\text{-}^{13}C^2}$ = 19Hz)
$\delta_{^{13}C^1=O}$:	196.4 ($J_{^{195}Pt\text{-}^{13}C^1}$ = ≲8 Hz)	195.9
$\delta_{^{13}C^3H_3}$:	22.3 ($J_{^{195}Pt\text{-}^{13}C^3}$ = 20 Hz)	--
$\delta_{^{195}Pt}$:	-73.6 ($J_{^{195}Pt\text{-}^{14}N}$ = 210 Hz, quintet)	-30.0 ($J_{^{195}Pt\text{-}^{14}N}$ = 230 Hz, quintet)

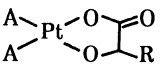
MS (m/z, MH^+)

317 (^{194}Pt, 33%), 318 (^{195}Pt, 34%)	303 (^{194}Pt, 33%), 304 (^{195}Pt, 34%)
319 (^{196}Pt, 25%)	305 (^{196}Pt, 25%), 307 (^{198}Pt, 7.2%)

Physical data for the new complexes

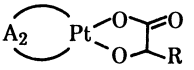
The data are summarized in Table 2.

Table 2. Physical data and recrystallization solvents for [Pt(OCOCHRO)A_2].

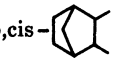


(A = R^1NH_2)

R^1	R	Recrystaln. solvent	MP (dp) °C	Yield %	Solubility (mg/ml H_2O)
H	H*	H_2O	130-	43	14
	CH_3	H_2O	150-	26	>100
	C_2H_5	---	180-185	28	>100
	$CH_2CH(CH_3)_2$	H_2O/EtOH	175-185	24	>100
	$(CH_2)_5CH_3$	---	165-175	30	80
	CH_2Cl	H_2O	145-	43	100
	CH_2OH	---	130-	45	>100
	C_6H_5	MeOH	185-	48	50
CH_3	H*	H_2O	170-	45	>100
	CH_2Cl	MeOH/acetone	hygroscopic	41	>100
	CH_2OH	MeOH/acetone	hygroscopic	51	>100
$(CH_3)_2CH$	H*	H_2O	200-	69	40



($A_2 = R_1^{NH_2}{}_{NH_2}$)

R^1	R	Recrystaln. solvent	MP (dp) °C	Yield %	Solubility (mg/ml H_2O)
$-CH_2-CH_2-$	H*	H_2O	165-	51	23
	CH_3	H_2O	205-	18	10
	CH_2Cl	H_2O	180-	30	NT
	C_6H_5	H_2O	240-	67	NT
RR- 	H*	H_2O	223-226	85	24
	CH_3	H_2O	180-230	13	>100
RS- 	H	H_2O/EtOH	217	32	>40
	CH_3	EtOH	220-	35	>100
	CH_2Cl	H_2O	199-	70	25
exo,cis- 	H	H_2O	225-	74	10
	CH_2OH	MeOH/acetone	200-	42	>50
	CH_2Cl	MeOH/acetone	200-	37	>100
	C_6H_5	MeOH	210-	42	100
	H	H_2O	220-	69	NT
	CH_2OH	MeOH/acetone	210-	56	10
	C_6H_5	MeOH	225-	60	5

NT: Not tested. * Ref. 2.

ANTITUMOR ACTIVITY

A series of diammine complexes [Pt(OCOCHRO)$(NH_3)_2$] was tested using Walker 256 carcinosarcoma, examining whether the substituents (R) influence the tumor inhibitory effect. As the size of the alkyl groups increases, the antitumor efficacy represented by the chemotherapeutic index (CI) increases up to the level of the ethyl derivative (C_2). However, introduction of alkyl groups bulkier than butyl (C_4) and other groups did not improve the CI value. These results are summarized in Table 3. The antitumor activity in vivo was also tested with respect to several other experimental tumor systems. Candidates selected for development were the two complexes, i.e., diammine(glycolato-O,O')platinum(II) ([Pt$(OCOCH_2O)$-$(NH_3)_2$], structure of 254-S, see Table 1) (1-3) and (R,R-1,2-di-

Table 3. Tumor inhibitory effect against Walker 256 carcinosarcoma.

[Pt(OCOCHRO)$(NH_3)_2$] R	ED_{50} (mg/kg)	LD_{50} (mg/kg)	CI	Solubility (mg/ml H_2O, 25°C)
H	3.8	31.6	8.3	14
CH_3 (L-lactato deriv.)	4.1 (5.4)	85.7 (92.5)	20.9 (17.1)	>100 (>100)
C_2H_5	4.0	108	27.0	>100
$CH_2CH(CH_3)_2$	5.2	70.7	13.6	>100
$(CH_2)_5CH_3$	11.1	141.4	12.7	80
CH_2Cl	4.8	100	20.8	100
CH_2OH	5.2	70.7	13.6	>100
C_6H_5	8.4	116.6	13.9	50

CI = LD_{50}/ED_{50}, Schedule: Sc-iv x 5 (Day 1 → 5), Host: Slc-Wistar rats.

Table 4. Comparison of CI* values of platinum complexes in the tumor panel.

System	Host	Schedule	CDDP	254-S	237
B16 (ip)	BDF_1 mice	(ip) day 1	10 (10/1)	8.3 (40/4.8)	6.1 (20/3.3)
L1210 (ip)	BDF_1 mice	(iv) days 1-5	1.5 (20/13)	1.4 (80/56)	3.3 (40/13)
		(ip) day 1	3.7 (10/2.7)	5.5 (40/7.2)	4.8 (20/4.2)
P388 (ip)	BDF_1 mice	(iv) days 1-5	1.4 (10/7)	3.2 (80/25)	2.3 (40/17)
		(iv) days 4-8	1.4 (13.5/9.8)	1.6 (78/50)	Inactive
Colon 38 (sc)	BDF_1 mice	(iv) days 1, 8	1.7 (31.1/18.3)	3.9 (113.6/29.2)	2.5 (42.4/17.3)
Walker 256 (sc)	Wistar rats	(iv) days 1-5	3.1 (5/1.6)	11.1 (20/1.8)	2.4 (5/2.1)
MX-1 (sc)**	BALB/C mice	(iv) days 1-5	2.9 (10.5/3.6)	6.3 (54.3/8.6)	2.3 (21.6/9.4)
L1210/CDDP (ip)	CDF_1 mice	(ip) day1	Inactive	Inactive	50 (10/ 0.2)

* CI = Optimal dose or max ILS/ILS_{30} or LD_{50}/ED_{50}.

** Evaluation: According to Battelle Columbus Laboratories Protocol. Nude mice

aminocyclohexane)(glycolato-O,O')platinum(II) ([Pt($OCOCH_2O$){R,R-1,2-$C_6H_{10}(NH_2)_2$}], 237) (2, 4). The comparative antitumor activities of these complexes are given in Table 4.

CONCLUSION

A new type of bidentate hydroxycarboxylic acid platinum(II) complexes was synthesized and characterized. Their in vivo antitumor activities were evaluated with transplantable tumor systems in rodents. Diammine(glycolato-O,O')platinum(II) (Code name 254-S) was finally selected as a candidate for clinical trials on the basis of its high activity against solid-form tumors and its low renal toxicity compared with cisplatin. 254-S is currently undergoing phase I study.

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STRUCTURE, MAGNETIC PROPERTIES AND ANTINEOPLASTIC ACTIVITY OF NOVEL PHTHALATO-BRIDGED COPPER(II) COMPLEXES

C.A. Tsipis, S.K. Shakhatreh, E.G. Bakalbassis, I.S. Pappas and A.S. Tsiftsoglou

INTRODUCTION

Several studies over the past years have established that a large variety of coordination compounds exert potent antineoplastic activity on a number of neoplasms (1). These findings have prompted us to search systematically for new and more potent metal containing antitumor agents. Our efforts have been directed towards the synthesis and biological study of copper(II) bi- and poly-nuclear complexes involving multiatom bridging units. Our rationale in this drug development has been as follows :

i. copper is a natural metal ion constituting polynuclear reaction sites in biological processes and it is thought to be essential for normal animal metabolism (2).

ii. copper(II) bi- and poly-nuclear complexes bridged by multiatom units exhibit a diverse range of interactions between the metal centers, which can be engineered by judiciously choosing the bridging and terminal ligands (3). Obviously, the establishment of any correlations between the nature and magnitude of these interactions and the biological activity of compounds, it is hoped to lead to new foundations for a molecular engineering of coordination compounds with biological properties forseable both in nature and magnitude.

In this paper we report on the preliminary results concerning the synthesis and structure elucidation of three novel (μ-phthalato)

copper(II) complexes,namely $|(dien)(ClO_4)Cu(\mu\text{-phth})Cu(dien)|(ClO_4)$, (1), $|(dien)Cu(\mu\text{-iphth})Cu(dien)|(ClO_4)_2$, (2) and $|(dien)Cu(\mu\text{-tphth})Cu(dien)|(ClO_4)_2$, (3) (where phth, iphth and tphth are the intervening phthalato, isophthalato and terephthalato ligands, respectively and dien the terminal diethylenetriamine ligand). Furthermore, we have determined the antineoplastic activity of these complexes on K-562 human myeloid leukemia and TE-671 human medulloblastoma cells in culture. The inhibitory potency of these agents was compared with that of the disodium salts of the free phthalato ligands, $Cu(ClO_4)_2$ and cis-platine under the same conditions.

MATERIALS AND METHODS

Starting materials.

All reagents and solvents were of analytical grade and were used without further purification.

Disodium salts of phthalic acid isomers were prepared by adding 2 mmoles of NaOH to an ethanol suspension of the corresponding phthalic acid (1 mmol). Addition of Et_2O resulted in the immediate precipitation of the white microcrystalline salt, which was filtered and dried.

Complex 1 was prepared by adding a methanol solution (25 ml) of 0.89 mmole of the phthalate piperidinium salt to a methanolic solution (25 ml) of 1.78 mmoles of $Cu(ClO_4)_2$ and 1.78 mmoles of the dien. The dark blue microcrystalline solid derived, upon recrystallization from hot methanol, gave single crystals proper for x-ray investigation.

Complexes 2 and 3 were prepared in a similar way, by using the general procedure described, previously (4). Single crystals proper for x-ray investigation were grown from the methanolic reaction mixture of 3. However, all attempts to grow single crystals for 2 have been unsuccessful, so far.

All agents with the exception of 3, were dissolved in dstd. water before added in cultures. 3 was first dissolved in DMSO and then diluted with dstd. water before added in cultures.

Optical and magnetic measurements.

They were carried out by using the same units and methods described (4) previously.

Cell cultures.

Cells used throughout this study were human myeloid leukemia K-562 and human medulloblastoma TE-671. These cells are grown and maintained in culture medium (RPMI-1640) supplemented with 10% fetal culf serum and antibiotics (streptomycin and penicillin). TE-671 cells are attached culture and are passed twice/weekly after trypsinisation. K-562 cells are grown in suspension culture under the same condition.

Determination of cell growth.

Cells were seeded at $1x10^5$ cells/ml (K-562) and $5x10^4$ cells/ml (TE-671) in the absence and presence of varying concentration of each agent for three days. By the end of this period, the cell number was determined microscopically (measurement with hemocytometer) and the degree of growth inhibition was assessed.

RESULTS AND DISCUSSION

Characterization of the Complexes.

The identification of compounds was based on elemental analysis and spectral studies (IR, UV-vis and EPR), and in the case of complexes 1 and 3, on x-ray data, as well.

A thorough analysis of the IR data provided information about the nature of the coordination sphere and the coordination number of each Cu(II) center of the complexes. Thus, all complexes exhibited the characteristic strong and broad $\nu_{as}(CO_2)$ and $\nu_s(CO_2)$ absorption bands due to the phthalato bridging units coordinated in an end-to-end fashion. However, the shape and the position of these bands were different for the three complexes, suggesting different coordination modes of the bridging ligand. Complexes 2 and 3 showed split $\nu_s(CO_2)$ bands, the two $\nu_{as}(CO_2)$-$\nu_s(CO_2)$ values being 218 and 193 and 206 and 162cm^{-1}, respectively. This calls for the coexistence of both bi- and anisobidentate carboxylato groups (5) in these complexes. Two bands were observed for 1, separated by ca.203cm^{-1}, suggesting an anisobidentate coordination mode for both carboxylato groups of the phthalato ligand. The absorption band associated with the ClO_4^- groups of all complexes were typical for ionic bonding mode (6). However, the broad absorption band at ca.1100cm^{-1} of complex 1 was broader and weakly split into three components. This calls for a weak unidentate coordination (6a)

of the ClO_4^- anion in 1. Moreover, all three complexes exhibited the characteristic bands along with their overtones due to the amino groups of the coordinated terminal dien ligand.

Insight concerning the stereochemistry of each copper center of the complexes was gained through the analysis of their electronic and EPR spectra. Complex 1 showed a broad-band envelope with a maximum at ca.16.10kK and a shoulder at ca.19.50kK. According to the well established electronic criteria for Cu(II) polyamine complexes (7) this spectrum indicates the coexistence of a square-pyramidal and a square-planar chromophore. The X-band spectrum of the complex-a single,slightly asymmetric derivative (g=2.083) at 295K- is in line with two Cu(II) ions in different environments (8).

2 exhibited an analogous ligand field spectrum-a broad- band envelope with a maximum at ca.16.10kK and a shoulder at ca.19.50kK.Therefore, a coexistence of both square-pyramidal and square-planar chromophores in the complex is very likely. Consistent with these stereochemistries was also the observed room-temperature axial powder EPR spectrum of the complex ($g_{\parallel}$=2.215, $g_{\perp}$=2.060; g_{av}=2.110), which calls for a |xy> ground state (9).

The ligand field spectrum of 3 -a broad band envelope with a maximum at ca.15.9kK and two shoulders at ca.19.20 and 12.80kK-strongly suggests the coexistence of both a square-planar and a five-coordinated Cu(II) chromophores. Consistent with these stereochemistries was also the observed room-temperature single somewhat asymmetric EPR spectrum of the complex (g=2.099).

The EPR spectra of all three complexes did not exhibit any sign of exchange interaction or triplet ground state.

Description of the structures.

Consistent with the proposed stereochemistries around each magnetic center were also, the crystallographic data (10,11) obtained for complexes 1 and 3.

The structure of 1 consisted of dimeric $|(dien)(ClO_4)Cu(1)(\mu\text{-phth})Cu(2)(dien)|^+$ cations and isolated perchlorate anions. An oxygen atom of a carboxylate group bridges two different Cu(2) centers (Cu(2)-O=2.009 Å and Cu(2)´-O=2.318 Å) of two different dimeric cations. Thus, infinite chains of dimeric cations are formed. The surrounding of Cu(1) was close to a square-base pyramid with one oxygen atom of

a perchlorate anion in the apical position (Cu(1)-O(ClO_4)=2.46 Å).The Cu(2) environment was close to a severely distorted octahedron or square planar.

The structure of 3 consisted also, of dimeric $|$(dien)Cu(μ-tphth) Cu(dien)$|^{2+}$ cations and isolated perchlorate anions. Two oxygen atoms of two different dimeric cations are coordinated (Cu-O(1)=1.97 Å and Cu-O(2)=2.47 Å) to each Cu(II) center. Thus, sheets of molecules of infinite dimeric cations are formed. The coordination geometry around each Cu(II) could be described as a distorted square-base pyramid.Its basal plane consisted of the three nitrogen atoms of a dien ligand and an oxygen atom (Cu-O(1)=1.97 Å) of a terephthalato bridging unit; one oxygen atom of another terephthalato ligand (Cu-O(2)=2.47 Å) occupied the apical position.

Magnetic Data.

Variable-temperature (4.2-295K) magnetic susceptibility data were collected for solid samples of all three Cu(II) complexes studied. These data were consistent with a chain structure. Two one-dimensional models- Ising (12) and Heisenberg (13)- of magnetic interactions were used to fit (10,11) these data. As a result a variety of magnetic exchange interactions, ranging from weak intramolecular ferromagnetic -intrachain exchange parameter for 1, J=5.22 cm^{-1}- to weak antiferromagnetic- J=-0.30 and -1.55 cm^{-1} for 2 and 3, respectively- ones were deduced. Moreover, the interchain exchange interactions were of weak antiferromagnetic type for all complexes.

Antineoplastic activity.

As shown in Fig.1A treatment of human leukemia K-562 cells in vitro with copper complexes caused a dose-dependent inhibition of cell proliferation which reached the max value (>60%) at 10^{-4}M. Comparatively, less inhibition of cell growth was obtained in TE-671 exposed in all three Cu(II) complexes. At 10^{-4} M all complexes caused only 25-30% inhibition of growth in TE-671 cells as compared to >60% in K-562 cells (Fig.1A,1B).

Although no substantial differences in antineoplastic activity were observed among the different copper complexes in either culture of neoplastic cells, careful examination indicated that 3 was relatively more inhibitory than 1 and 2 complexes at low equal molar concentrations (Fig.1). These data suggest that these complexes are mo-

re potent inhibitors of cell growth in leukemias rather neurogical tumors. Furthermore, the data illustrated in Table 1, indicate that (μ-phthalato) Cu(II) complexes were relatively less potent inhibitors of

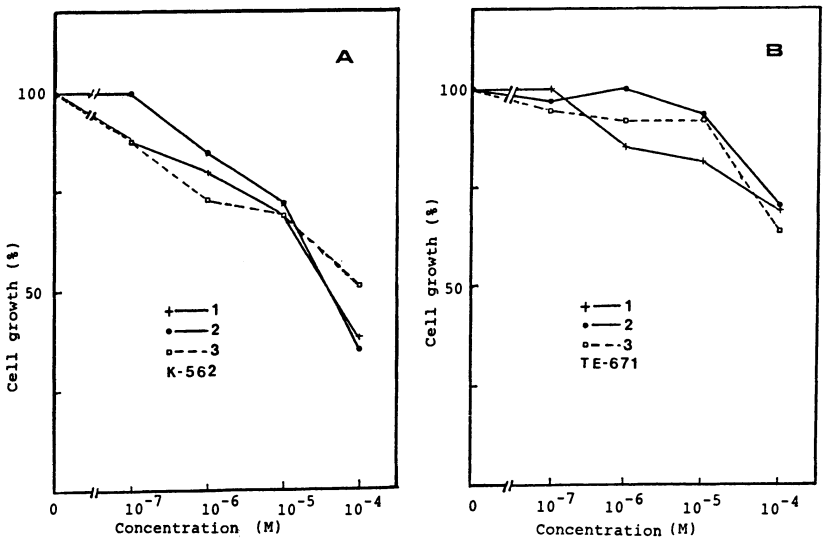

Figure 1. Dose-dependent effects of treatment of human leukemia K-562 and human medulloblastoma TE-671 with (μ-phthalato) copper(II) complexes in culture. Cells were seeded at $1x10^5$ cells/ml (A, K-562) and at $5x10^4$ cells/ml (B, TE-671) in the absence and in the presence of varying concentrations of each complex (1,2 and 3, see text). Three days following plating, cell number was determined with the use of a hemocytometer. Cell growth was assessed as per cent of growth of untreated cells.

cell proliferation as compared to cis-platine, a known potent antineoplastic metal-organic compound. The observation, however, that none of the free bridging ligands (phthalato, isophthalato, terephthalato) inhibited cell growth, while treatment with $Cu(ClO_4)_2$ caused a substantial degree of inhibition (∿40%) of cell growth, tend to suggest that the presence of Cu(II) ions might be responsible for the antineoplastic activity of the μ-phthalato Cu(II) complexes. Accordingly, it is possible that the biologically active species could be the $|Cu(dien)|^{2+}$ ones, resulting from the dissociation of the complexes at very low molar concentrations. Such d^9-ML_3 fragments could interact as active species on cellular elements including DNA, RNA and

Table 1. Antineoplastic activity of (μ- phthalato) Cu(II) complexes in cultures of K-562 and TE-671 neoplastic cells.

Treatment	Conc. (M)	K-562 Human leukemia cells	TE-671 Human medulloblastoma cells
		Cell growth (% of control)	Cell growth (% of control)
None	—	100(0)[a]	100(0)
$PhthNa_2$	10^{-4}	99(1)	97(3)
1	10^{-4}	38(62)	69(31)
	10^{-5}	69(31)	82(18)
	10^{-6}	80(20)	86(14)
	10^{-7}	88(12)	100(0)
Cis-Platine	10^{-5}	25(75)	N.D
	10^{-6}	71(29)	N.D
$iphthNa_2$	10^{-4}	92(8)	122(+22)
2	10^{-4}	35(65)	70(30)
	10^{-5}	72(28)	94(6)
	10^{-6}	85(15)	100(0)
	10^{-7}	103(+3)	97(3)
Cis-Platine	10^{-5}	13(87)	N.D
	10^{-6}	60(40)	N.D
$tphthNa_2$	10^{-4}	102(+2)	104(+4)
3	10^{-4}	51(49)	64(36)
	10^{-5}	69(31)	93(7)
	10^{-6}	73(27)	92(8)
	10^{-7}	88(12)	95(5)
Cis-Platine	10^{-5}	10(90)	N.D
	10^{-6}	56(44)	N.D
$Cu(ClO_4)_2$	10^{-4}	59(41)	77(23)

(a) The numbers in parentheses indicate the degree of inhibition of cell growth as compared to non-treated cells. (+) Indicates increase in cell growth. N.D:Not determined. See text under Materials and Methods for experimental details.

proteins as well, which are of fundamental importance in cell proliferation. As a consequence, the small differences observed in the antineoplastic activity of the complexes could be correlated with the small differences in their instability constants. Finally, although the antiferromagnetic complexes appear to be more potent antineoplastic agents than the ferromagnetic one, a general conclusion could not be drawn on the basis of these preliminary results only.

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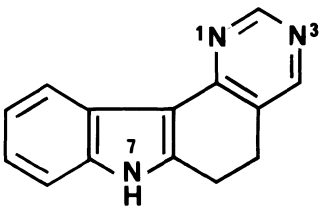
SYNTHESIS AND STRUCTURE OF THE TETRA 5,6-DIHYDROPYRIMIDINO[5,4-c] CARBAZOLE DICHLORIDE HEXACHLOROPLATINATE (IV) TETRAHYDRATE

B. Viossat, Nguyen-Huy Dung, J.C. Lancelot and M. Robba

INTRODUCTION

The derivatives of 5,6-dihydropyrimidino[5,4-c] carbazole (called hereafter DPC) with antineoplastic intention and synthesized from the design of ellipticine showed activity in the course of preliminary study on mouse leukemia P388. In light of the properties and crystal structure of [ethidium]$_2$ $PtCl_4$ (1) and of [rhodamine 123]$_2$ $PtCl_4.H_2O$ (2), the first tetrachloroplatinate (II) with anticancer activity, we attempt to synthesize an analogous complex salt by replacing the rhodamine cation by the protonated DPC molecule ($DPCH^+$).



The DPC molecule is a tetraheterocycle in which the cyclohexadiene ring is in a skew chair conformation. Moreover, it exhibits a dimorphism as revealed by X-ray crystallographic studies (3,4).

MATERIALS AND METHODS

Synthesis.

DPC (5.10^{-4} mol) was dissolved in HCl 1 M solution (25 ml). To this solution was added K_2PtCl_4 ($2.5\ 10^{-4}$ mol) dissolved in water (25 ml). A yellow precipitate formed immediately. This precipitate was partially dissolved in HCl 1 M solution.

Single crystals were obtained by slow evaporation of this solution under ambiant air pressure. In these conditions, there is a slow oxidation of Pt(II) to Pt(IV), as confirmed by X-ray study.

Crystal data.

$a = 8.222(3)$, $b = 13.140(2)$, $c = 14.753(2)$ Å ; $\alpha = 114.81(1)$, $\beta = 92.93(2)$, $\gamma = 95.54(2)°$. $V = 1433$ Å^3. $M_r = 1439{,}8$. $\rho_{obs} = 1.67$, $\rho_{cal} = 1.67$ g.cm^{-3}. Space group : triclinic $P\bar{1}$. $Z = 1$.
Unit-cell content : 1 $[PtCl_6^{2-}]$; 2 Cl^- ; 4 H_2O solvate molecules ; 4 $DPCH^+$ (i-e : 2 crystallographically independent cations).
Formula $[C_{14}N_3H_{12}^+]_4\ [PtCl_6^{2-}]\ 2\ Cl^-.4\ H_2O$
$C_{56}H_{56}Cl_8N_{12}O_4Pt$

Solution of the structure.

The platinum atom is in the centre of symmetry. The chloride ions were found from a three-dimensional Patterson synthesis and subsequent least-squares refinement and electron density difference syntheses revealed all the non-hydrogen atoms. Further refinement using a full-matrix least-squares and all atoms with anisotropic temperature factors minimized $\Sigma\ \omega\ (|F_o - F_c|)^2$. Final $R = Rw = 0.028$ on the basis of 5044 independent reflections.

RESULTS

Platinum environment.

The platinum (IV) atom in the centre of symmetry exhibits a slightly distorted octahedron coordination. The Pt (IV)-Cl distances of 2.311(1), 2.320(2) and 2.325(1) Å as well as the Cl-Pt-Cl angle values, very close to 90° (the maximum deviation is less than 1.27(5)°) agree well with those found in K_2PtCl_6 (5). As shown in Fig. 1, there is no evidence for coordination of $DPCH^+$ to platinum

(IV) : thus, there is a salt formation as indicated by the crystal structure.

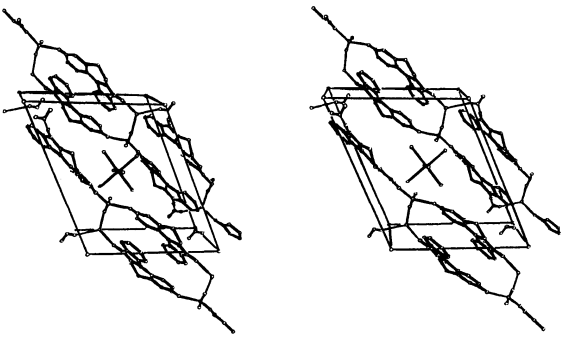


Fig. 1 Stereoscopic view of the unit-cell showing the octahedral coordination around Pt (IV) and the intermolecular bonding.

$DPCH^+$ conformation.

The two independent $DPCH^+$ cations are labelled from N(1) to C(17) and N(21) to C(37) respectively.

In the $DPCH^+$ cation, the cyclohexadiene ring remains in a skew chair conformation. The other rings (benzene, indole and pyrimidine) are as expected from previous X-ray studies.

It is noteworthy that the two independent DPC molecules are protonated on the two homologous sp^2 N(3) and N(23) atoms respectively. As a matter of fact, the lone pair of N(1) atom is lesser donor than the homologous pair of the N(3) atom, in consequence of the steric hindrance due to the vicinity of the hydrogen atom bonded to the C(11) atom. The donor capacity of this N(3) or N(23) atom lone pair was observed in the coordination complex between $Co(No_3)_2$ and DPC molecule where a covalent Co(II)-N(3) bond was found (6).

Intermolecular bonding

The packing of the title compound is shown in Figure 1.

In addition to the cation-anion charge interactions, there is an extensive hydrogen-bonding network stabilizing the crystal.

The crystal	Distance(Å)	Symmetry code
Cl(4) .... HB(01)-O(1)	3.051(4)	i : 1-x, 1-y, 1-z
Cl(4) .... HB(02^{ii})-O(2^{ii})	3.125(7)	ii : x , 1+y, z
Cl(4) .... H(N7^{iii})-N(7^{iii})	3.115(5)	iii :-1+x, y , 1+z
O(1) .... H(N3^{i})-N(3^{i})	2.842(7)	iv : -x , 1-y, 1-z
O(1) .... H(N23)-N(23)	2.745(7)	
O(2) .... H(N27^{iv})-N(27^{iv})	2.724(8)	

Thus, Cl(4) is bonded to the two H_2O molecules and one $DPCH^+$ via the N(7^{iii}) atom. Fig. shows that one $DPCH^+$ cation is linked by hydrogen-bonding to a first $DPCH^+$ (crystallographically independent from the previous one) via the O(1) atom of $H_2O(1)$ and to a second $DPCH^+$ via the O(2) atom of $H_2O(2)$ and the Cl(4) ion ; thus forming an infinite zigzag chain. Two infinite chains related by a centre of symmetry are bridged by O(1) and Cl(4) atoms giving rise to a two-dimensional bandlike network.

Work is in progress to further characterize the biological properties of this and related compounds.

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ANTITUMOR ACTIVITY OF ZINDOXIFENE DERIVED PLATINUM COMPLEXES

E. Von Angerer, N. Knebel, H. Schönenberger and J. Engel

Based on the new antiestrogen zindoxifene (1), cis-diaminodichloroplatinum(II) complexes were synthesized. Two of these complexes (1-Pt, 2-Pt) were studied in detail (Fig. 1). They showed high binding affinities for the estrogen receptor (RBA = 6.5 and 4.4 resp.; E2: RBA = 100) but were devoid of estrogenic or antiestrogenic activity in the mouse uterine weight test. Antineoplastic activity was evaluated in transplanted MXT mouse mammary tumors. The growth of hormone-independent tumors was not or only weakly inhibited, but both complexes showed a marked inhibition of hormone-sensitive tumors with T/C-values (mean tumor weight) between 11 and 23 % following the administration of 3 x 20 mg/kg/week for 6 weeks. The free ligands of the complexes were much less active. No cytostatic activity of the complexes was found in vitro with estrogen receptor negative human MDA-MB 231 breast cancer cells. However, the growth of hormone-dependent MCF-7 breast cancer cells was inhibited: Cell numbers were decreased by 52 % and 66 % resp. at a concentration of 5×10^{-6} molar of the complex. These results make a specific mode of action involving the estrogen receptor likely.

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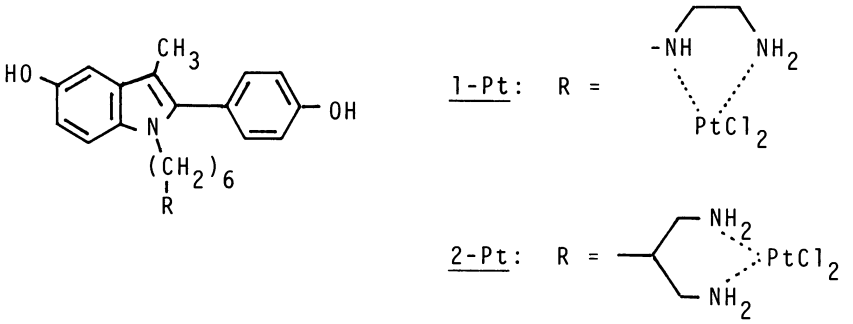

Fig. 1: Chemical structures of platinum complexes binding to the estrogen receptor

PLATINUM (II) COMPLEXES WITH AMINOPYRIMIDINES

K.I. Yakovlev, A.I. Stetsenko, G.M. Alekseyeva, A.A. Tulub, L.B. Selderkhanova and I. Yu. Mamelkina

Triamine platinum(II) complexes with aminooxypyrimidines - cis-$[Pt(NH_3)_2LX]X$, where L - cytosine(Cyt), cytidine(Cyd), isocytosine(i-Cyt), X - Cl, Br, NO_2, as well as isomeric non-electrolyte Pt(II) complexes with 2-aminopyrimidine and its 4-methyl and 4-methoxy derivatives - $[PtL_2X_2]$ ($X - Cl^-$, Br^-, I^-) have been synthesized and identified [1,2] .

The complexes were obtained by reactions:

$$cis\text{-}[Pt(NH_3)_2X_2] + L \xrightarrow[H_2O]{90^\circ C} cis\text{-}[Pt(NH_3)_2LX]X \quad (1)$$

$$K_2[PtX_4] + 2L \xrightarrow[H_2O]{20^\circ C} cis\text{-}[PtL_2X_2] + 2KX \quad (2)$$

$$cis\text{-}[PtL_2X_2] \xrightarrow[DMSO]{70^\circ C} trans\text{-}[PtL_2X_2] \quad (3)$$

The composition and coordination formulas of the compounds have been established by elementary analysis and measurements of molar electrical conduction. All complexes contain neutral molecules of aminopyrimidines with monodentate coordination.

The geometric configuration of non-electrolyte complexes with 2-aminopyrimidines has been revealed by the method of long-wave IR spectroscopy. In the spectra for cis-isomers doublet absorption bands 330-340 cm^{-1}(Pt-Cl) 231-249 cm^{-1}(Pt-Br), 202-218 cm^{-1}(Pt-I) have been observed in the region of stretching vibrations of Pt-X bonds whereas in those for trans-compounds narrow singlet bands corresponding to ν_{as} (Pt-X) have been detected.

It is evident from the ^{1}H-NMR spectra of free ligands and complexes in DMSO-d_6 that coordination of aminopyrimidines by Pt(II) is accompanied by a downfield shift of non-labile proton signals. For cytosine and its derivatives the shift of the signal of proton H(5) is stronger($\Delta\delta$ H(5) = 0.20 - 0.28 p.p.m.) compared with the proton H(6)($\Delta\delta$ H(6) = 0.07 - 0.15 p.p.m.), which allows to infert the participation of the heteroatom of nitrogen N(3) in the bond with the central atom. The same conclusion is drawn from the spectra ^{13}C-NMR in which the largest changes are observed for the signals of carbon atoms C(2) and C(4) which are closest to the donor atom N(3).

For substituted 2-aminopyrimidines the strongest downfield for the signal H(6)($\Delta\delta$ H(6) = 0.56 - 0.64 p.p.m.,$\Delta\delta$ H(5) = 0.11 - 0.15 p.p.m.), it being attended by spin-spin interactions of the proton H(6) with the magnetic nucleous of the isotope ^{195}Pt(^{3}J ^{195}Pt-H(6) = 36-40 Hz). This is evidence of the formation of the bond Pt-N(1) atom.

The strong downfield shift of the singlet signal of aminogroup protons($\Delta\delta$ NH_2 = 1.0 - 1.8 p.p.m.) on coordination and its splitting into a doublet is accounted for by the formation of a structure in which the positive charge is partially localised on the nitrogen atom of amino-group. In this case the free rotation about the bond C-NH_2 is retarted and the protons of NH_2-group are no longer equivalent.

The oxidation state of Pt in cis-$[Pt(NH_3)_2CytCl]Cl$ is (2+), which follows from the value $E_{Pt\ 4f\ 7/2}$ = 72.8 eV. At the same time in the ESR spectra of triamines with Cyt and Cyd weak signals($g_{\parallel}$ = 1.97; $g_{\perp}$ = 2.41) are observed, which is evidence from the existence of paramagnetic centures.

The coordination of Cyt and i-Cyt leads to a 700-

fold and 300-fold enhancement, respectively, of their acidic properties. The influence of the intrasphere ligand on the acidic properties of complexes decreases in the order: Cl > Br > NO_2.

The kinetics of interactions between cis-$[Pt(NH_3)_2$ CydX$]X$ (X = Cl,Br) and Cyd, guanosine(Gua) and inosine (Ino)

$$[Pt(NH_3)_2CydX]^+ + L \xrightarrow{K_{obs.}} [Pt(NH_3)_2CydL]^{2+} + X^- \quad (4)$$

has been studied conductometrically under conditions of the pseudofirst order(5-50-fold excess of the entering ligand) at 50° and 60° [3] .

The nature of the entering nucleoside and the galogenide-ion being substituted influences the reaction rate. The value of $K_{obs.}$ decreases in the order Cyd > Gua ≈ Ino and increases on the replacemrnt of Cl^- by Br^-(table 1).

Table 1. The values of $K_{obs.}$ at [L] /[Pt] = 50

Complex	Entering ligand,L	t °C	[L] M	$K_{obs.}$ 10^5 sec^{-1}
cis- $Pt(NH_3)_2CydCl$ Cl	Cyd	50	$2.5 \cdot 10^{-2}$	17.7 ± 0.6
	Ino	-	-	13.9 ± 0.5
cis- $Pt(NH_3)_2CydBr$ Br	Cyd	-	-	25.3 ± 0.8
	Ino	-	-	18.9 ± 0.8
cis- $Pt(NH_3)_2CydCl$ Cl	Cyd	60	$1.25 \cdot 10^{-2}$	43.4 ± 0.2
	Ino	-	-	37.3 ± 0.3
	Gua	-	-	34.5 ± 0.2

In the reactions between triamines and Cyd the value $K_{obs.}$ is linearly dependent on ligand concentration

$$K_{obs.} = K_1 + K_2[L] \ , \quad (5)$$

where K_1 corresponds to the hydrolytic route, K_2 - to the nucleophilic replacement according to a second-order reaction.

On the entering of Gua and Ino $K_{obs.}$ is non-linear-

ly dependent on L and decreases with increased concentration of X. For this case we have proposed a scheme of the substitution process reflecting the competitive entrance of the ligand and halogenide-ion into the aqua complex

$$[Pt(NH_3)_2CydX]^+ + L \xrightarrow{K_2} [Pt(NH_3)_2CydL]^{2+} + X^- \quad (6)$$

$$[Pt(NH_3)_2CydX]^+ + H_2O \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [Pt(NH_3)_2CydH_2O]^{2+} + X^- \quad (7)$$

$$[Pt(NH_3)_2CydH_2O] + L \xrightarrow{K_3} [Pt(NH_3)_2CydL]^{2+} + H_2O \quad (8)$$

According to the scheme suggested and taking into account the stationary state of the intermediate aqua-complex the expression for $K_{obs.}$ under conditions of pseudofirst order takes the form

$$K_{obs.} = K_2[L] + \frac{K_1 K_3 [L]}{K_{-1}[X] + K_3 [L]} \quad (9)$$

The contribution of the S_{N2} route in reaction with Gua and Ino is very small - 3-5% at a 50-fold excess of the ligand. For the interaction with Cyd under the same conditions the contribution of S_{N2} is 25-40%.

Quantum chemical calculations of the electron structure of the complexes cis-$[Pt(NH_3)_2CytCl]^+$ and cis-$[Pt(NH_3)_2(i\text{-}Cyt)Cl]^+$ have been carried out by the SCF MO LCAO method in approximation of CNDO/S3.

The calculated and experimental values of the oscillator forces and wave lengths corresponding to the maximum absorption in the experimental spectra over the range 185-350 nm are in good agreement. The best agreement of experimental and calculated spectra is observed when Cyt and i-Cyt ligands are turned perpendecular to the coordination plane.

The electron absorption spectrum over the range $\lambda <$ 230 nm is entirely due to the transfers between occupied and virtual MO of Cyt and i-Cyt ligands. In the long-wave region there are bands corresponding to the transfer of electron density from the chlorine atom to the Pt $5d_{x^2-y^2}$ atomic orbital and between of five d-orbi-

tals of the Pt.

METHODS

The IR spectra were recorded on "Specord-75 IR" spectrophotometer and the ^{1}H-NMR spectra - on "Tesla BS-487C, 80 MHz". The pH and conductometric measurements were performed using a "pH-121" pH-meter and CD-1 conductometer. The ESR spectra were measured on "Bruker, 100 KHz" spectrometer at 77°K.

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