Program and Abstracts

The Sixteenth International Conference on Antiviral Research

Sponsored By

The International Society For Antiviral Research

Savannah Marriott Riverfront Hotel

Savannah, Ga., USA

April 27–May 1, 2003

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Organization

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and
Sixteenth International Conference on Antiviral Research

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Introduction To Sponsor

The International Society For Antiviral Research (ISAR)

The Society was organized in 1987 as a non-profit scientific organization for the purpose of advancing and disseminating knowledge in all areas of antiviral research. To achieve this objective, the Society organizes an annual meeting. The Society is now in its fifteenth year of existence, and has about 800 members representing 30 countries. For membership application forms or further information, please contact Dr. Brent E. Korba, Secretary, ISAR; Georgetown University, DMVI, 13 Taft Court, Suite 101, Rockville, MD 20850; Ph. (301) 309-6145 Ext. 27; Fax (301) 309-1553; E-mail korbabe@gusun.georgetown.edu. Membership application forms will also be available at the Conference Registration desk, or from our website www.isar-icar.com.

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Additional support provided by

Office of AIDS Research, National Institutes of Health, Bethesda, Md., USA

Satellite Symposium

Clinical Update on Antiviral Drugs

Sunday, April 27, 2003, 1 p.m. to 6 p.m.
Savannah Marriott Riverfront Hotel

Reception, 6 p.m. to 8 p.m.
Savannah Marriott Riverfront Hotel

Social Functions:

Opening Reception:

Monday, April 28, 2003, 7:30 p.m. to 10 p.m.
Savannah Queen Riverboat Cruise
River Street Dock

Conference Banquet:

Wednesday, April 30, 2003, 7 p.m. to 10 p.m.
Savannah Marriott Riverfront Hotel

Final Program

Sixteenth International Conference on Antiviral Research

Sponsored by the
International Society for Antiviral Research

Savannah Marriott Riverfront Hotel
Savannah, Ga., USA

April 27 – May 1, 2003

Monday, April 28

Oral Session I: Retroviruses

Chairpersons: Jan Balzarini, John C. Drach

8:30am Welcome–John C. Drach, President, ISAR
Earl R. Kern, Chair, ISAR Conference Committee

8:45 Plenary Speaker
Beatrice Hahn, The University of Alabama at Birmingham, Birmingham, Ala., USA
"Origins and Evolution of Primate Lentiviruses"

9:15 1. "Lock-In" Modified cycloSal-D4TMP and BVDUMP Triesters - Extension of a Pronucleotide System.
C. Meier, M. Ruppel, D. Vukodinovic, A. Meerbach, P. Wutzler, E. De Clercq, and J. Balzarini.
University of Hamburg, Hamburg; University of Jena, Jena, Germany; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

9:30 2. Anti-HIV Activity Profile of AMD070, an Orally Bioavailable CXCR4 Antagonist.
D. Schols, S. Claes, S. Hatse, K. Princen, K. Vermeire, E. De Clercq, R. Skerlj, G. Bridger, and G. Calandra.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; and AnorMED, Inc., Langley, B.C., Canada.

9:45 3. Persistence of Antiretroviral Activity in Chronically Infected Cells Following a Short-Term Exposure to HIV-1 Protease Inhibitors (PIs).
A.S. Mulato, G.-X. He, E. Eisenberg, and T. Cihlar.
Gilead Sciences, Foster City, Calif., USA.

10:00 Break

10:30 4. Structure-Function Relationship of New Aminoglycoside-Arginine Conjugates (AAC) as Inhibitors of HIV-1 Entry Step.
A. Lapidot, V. Vijayabaskar, H.H. Lara, A. Kalinkovich, and G. Borkow.
Weizmann Institute of Science, Rehovot, Israel.

10:45 5. Cellular Factors Involved in the Acquisition of Drug-Resistance to Antiretroviral Drugs.
F. Dianzani, O. Turriziani, J.D. Schuetz, I. Solimeo, F. Bambacioni, A. Maffeo, O. Butera, and G. Antonelli.
University "Campus Biomedico"; University "La Sapienza", Rome, Italy; and St. Jude Children's Research Hospital, Memphis, Tenn., USA.

11:00 6. Resistance Analyses of Virologic Failure Through 48 Weeks Among Treatment-Naïve Patients Taking Tenofovir DF (TDF) or Stavudine (d4T) with Lamivudine (3TC) and Efavirenz (EFV).
D.J. McColl, N.A. Margot, S. Tran, D.F. Coakley, A.K. Cheng, and M.D. Miller.
Gilead Sciences, Inc., Foster City, Calif., USA.

11:15 7. A Phase IIB Prospective, Randomized, Controlled Study Evaluating Ampligen During Structured Treatment Interruption (STI) of HAART in HIV Infection.
W. Mitchell, G. Blick, D. Strayer, W. Carter, and AMP 720 Investigators.
Vanderbilt University, Nashville, Tenn.; Circle Medical, Norwalk, Conn.; and Hemispherx Biopharma, Philadelphia, Pa., USA.

11:30 Plenary Speaker – Elion Award
John C. Martin, Gilead Sciences, Inc., Foster City, Calif., USA
"A Brief History of Nucleotide Antivirals"

12:00 Lunch

Monday, April 28

Oral Session II: Hepadnaviruses, Retroviruses

Chairpersons: Timothy Block, Joseph M. Colacino

1:30pm Plenary Speaker
Gilles Gosselin, University of Montpellier, Montpellier, France
"β-L-2'-Deoxythymidine and 3'-O-L-valinyl Ester of β-L-2'-deoxycytidine: Two Potent and Selective Anti-HBV Drugs"

2:00 8. A Novel Phenotypic Assay for Monitoring HBV Drug Resistance During Antiviral Treatment.
D. Durantel, S. Durantel, C. Pichoud, B. Werle, M.N. Brunelle, C. Trépo, and F. Zoulim.
INSERM, Lyon, France.

2:15 9. Synthetic and Natural Glycolipids Induce 2-5-OA Synthetase Transcripts and have Direct Antiviral Activity Against Hepatitis B Virus (HBV).
A.S. Mehta, B. Conyers, X. Lu, S. Ouzonov, E. Simsek, R.A. Dwek, and T.M. Block.
Thomas Jefferson University, Doylestown, Pa., USA; and University of Oxford, Oxford, U.K.

2:30 10. Characterization and Clinical Significance of Lamivudine Resistant Hepatitis B Virus (HBV) Mutations in Human Immunodeficiency Virus (HIV) and HBV Co-Infected Individuals.
A. Bartholomeusz, S. Locarnini, A. Ayres, L. Cooley, S. Lewin, S. Crowe, A. Mijch, S. Roberts, and J. Sasadeusz.
Victorian Infectious Diseases Reference Laboratory, North Melbourne, Vic.; Victorian Infectious Diseases Service, Parkville, Vic.; The Alfred Hospital, Prahran, Vic.; and The Burnet Institute, Prahran, Vic., Australia.

2:45 11. Identification of Pre-Treatment HBV DNA Markers that are Predictive of Response to Lamivudine Therapy in Patients Infected with HBV Precore Variants.
B. Korba, A. Smedile, M. Rizzetto, M. Lagget, J. Gerin, and A. Ciancio.
Georgetown University, Rockville, Md., USA; Ospedale S. Giovanni Battista di Torino, Italy.

3:00 12. Resistance Surveillance in HBeAg- Chronic Hepatitis B (CHB) Patients Treated with Adefovir Dipivoxil (ADV) for Two Years.
C.S. Gibbs, S. Xiong, H. Yang, C.E. Westland, W.E. Delaney IV, D. Colledge, A. Bartholomeusz, V. Thibault, Y. Benhamou, P. Angus, M. Wulfsohn, J. Fry, C.L. Brosgart, and S. Locarnini.
Gilead Sciences, Foster City, Calif., USA; VIDRL, North Melbourne, Australia; Hospital Pitie-Salpetriere, Paris, France; and Austin and Repatriation Medical Center, Heidelberg, Australia.

3:15 13. Quantitative Analyses of Hepatic HBV cccDNA During the Natural History of Chronic Hepatitis B and Adefovir Dipivoxil Therapy: Results of an International Multicenter Study.
F. Zoulim, B. Werle, K. Wursthorn, J. Petersen, S. Bowden, S. Locarnini, G. Lau, C. Brosgart, S. Xiong, W. Delaney IV, and C. Gibbs.
INSERM, Lyon, France; Universitaetsklinikum, Hamburg-Eppendorf, Germany; VIDRL N. Melbourne, Australia; Queen Mary Hospital, Hong Kong; and Gilead Sciences, Foster City, Calif., USA.

3:30 14. RNA Interference of HIV Coreceptors.
J.A. Este, A. Gutierrez, N. Tapia, M. Armand-Ugon, J. Blanco, J. Gomez, B. Clotet, and M. Martinez.
Universitat Autònoma de Barcelona, Barcelona, Spain.

3:45 15. Specific Silencing of HIV-1 Env Gene Using Small Interfering RNAs in Mammalian Cells.
H. Takaku, W.-S. Park, N. Miyano-Kurosaki, and M. Hayafune.
Chiba Institute of Technology, Chiba, Japan.

Monday, April 28

Poster Session I: Retroviruses, Hepadnaviruses

4 p.m. – 6 p.m.

16. Synthesis and Anti-HIV Activities of L-β-3'-Fluoro-2'-3'-dideoxy-2',3'-didehydro-4'-thio-nucleosides.
    W. Zhu, R.F. Schinazi, and C.K. Chu.
    The University of Georgia, Athens; and Emory University School of Medicine/Veterans Affairs Medical Center, Decatur, Ga., USA.

17. Structure-Activity Relationships of D- and L-3'-Fluoro-2',3'-Unsaturated Nucleosides as Anti-HIV Agents.
    G. Gumina, Y. Chong, W. Zhou, R.F. Schinazi, and C.K. Chu.
    The University of Georgia, Athens; and Emory University School of Medicine/Veterans Affairs Medical Center, Decatur, Ga., USA.

18. Anti-HIV Activity and Molecular Mechanism of Drug Resistance of D- and L- 2'-F-4'-S-d4N.
    H. Choo, Y. Chong, R.F. Schinazi, and C.K. Chu.
    The University of Georgia, Athens; and Emory University School of Medicine/Veterans Affairs Medical Center, Decatur, Ga., USA.

19. Glycopeptide Antibiotic Derivatives Selectively Inhibit Human Immunodeficiency Virus Replication in Cell Culture.
    J. Balzarini, C. Pannecouque, S. Hatse, D. Schols, E. De Clercq, Y. Pavlov, S.S. Printsevskaya, and M. Preobrazhenskaya.
    Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; and Russian Academy of Medical Sciences, Moscow, Russia.

20. Abacavir Phosphoramidate Analogues: The Effect of Amino Acid Variations on the Biological Activity.
    A. Brancale, S. Srinivasan, A. Hassan-Abdallah, Y. Wang, R. Pathirana, A. Siddiqui, S. Harris, C. McGuigan, E. De Clercq, J. Balzarini, K. Gudmundsson, and S. Daluge.
    Cardiff University, Cardiff, U.K.; Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; and GlaxoSmithKline, Research Triangle Park, N.C., USA.

21. In Vitro and In Vivo Activity of a Novel Small Molecule Against HIV.
    L.S. Kucera, N.P. Iyer, S.L. Morris-Natschke, K.S. Ishaq, and R.A. Fleming.
    Wake Forest University School of Medicine, Winston-Salem; University of North Carolina, Chapel Hill; and Kucera Pharmaceutical Co., Winston-Salem, N.C.; USA.

22. Dinucleotide Inhibitors of HIV Integrase: Implications of Inhibitor Structure and Stereochemistry.
    V. Nair, B.I. Seo, and G. Chi.
    University of Georgia, Athens, Ga., USA.

23. Anti-HIV Activity of Newly Cloned Secretory Leukocyte Protease Inhibitor (SLPI).
K. Konopka, N.R. Shine, S.C. Wang, N. Duzgunes, and C.P. Whitman.
University of the Pacific, San Francisco, Calif.; and University of Texas at Austin, Austin, Tex., USA.

24. Synthesis of Anti-HIV CADA Compounds and Quantitative Structure-Activity Relationships for CD4 Down-Modulation.
T.W. Bell, K. Dey, Q. Jin, M.F. Samala, A. Sodoma, K. Vermeire, E. De Clercq, and D. Schols.
University of Nevada, Reno, Nevada, USA; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

25. New Pyrophosphate Analogues as Substrates and Inhibitors of HIV Reverse Transcriptase.
M.K. Kukhanova, N.F. Zakirova, A.V. Ivanov, M.V. Yasko, Yu.S. Skoblov, and A.R. Khomutov.
Engelhardt Institute of Molecular Biology, Moscow, Russia.

26. The Potential of Novel Ribonucleotide Reductase Inhibitors, Didox and Trimidox, Compared to Hydroxyurea to Enhance NRTI Antiviral Activity In Vivo.
R. Sumpter, H. Elford, M. Inayat, T. Sugg, P. Tsai, and V. Gallicchio.
University of Kentucky, Lexington, Ky.; and Molecules for Health, Inc., Richmond, Va., USA.

27. 1-Substituted 4-Methoxy-2-phenylbenzimidazoles as NNRTIs: Synthesis and Anti-HIV Evaluation.
G. Pürstinger, A. Groβ, A. Neulinger, M. Witvrouw, C. Pannecouque, J. Balzarini, and E. De Clercq.
Universität Innsbruck, Innsbruck, Austria; Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium

28. 3,4-Dialkyoxyquinolin-2(1H)-ones and 4-Alkyoxy-1,6-naphthyridin-2(1H)-ones as HIV Nonnucleoside RT Inhibitors.
G. Freeman, A. Hopkins, C. Andrews III, G. Lowell, S. Gonzales, J. Cowan, L. Schaller, G. Koszalka, R. Hazen, L. Boone, R. Ferris, K. Creech, G. Roberts, S. Short, K. Weaver, J. Milton, D. Porter, D. Stuart, D. Stammers, and J. Chan.
GlaxoSmithKline Research and Development, Research Triangle Park, N.C.; University of Oxford, Oxford, U.K.; Pfizer Central Research, Sandwich, Kent, U.K.; Rush University, Chicago, Ill., and Array Biopharma, Boulder, Colo., USA.

29. Characterization of a Novel Series of Nevirapine-like Next-Generation NNRTIs with Broad Antiviral Potency Against NNRTI-Resistant HIV.
P.R. Bonneau, L. Doyon, J. Duan, B. Simoneau, C. Yoakim, R. Déziel, W. Ogilvie, L. Bourgon, M. Garneau, F. Liard, C. Plouffe, S. Tremblay, E. Wardrop, M. Bös, and M.G. Cordingley.
Boehringer Ingelheim (Canada), Ltd., Laval, Quebec, Canada.

30. Pharmacokinetics and Developability of GW4511, A Novel Benzophenone Non-Nucleoside Reverse Transcriptase Inhibitor of HIV-1.
D. Reynolds, P. Wheelan, C. Edwards, J. Tidwell, J. Chan, G. Freeman, K. Romines, L. Schaller, J. Cowan, R. Ferris, D. Hazen, M. St. Clair, and L. Boone.
GlaxoSmithKline, Research Triangle Park, N.C., USA.

31. Prevention of HIV-1 Infection by Phthalocyanines.
A.N. Vzorov, L.G. Marzilli, R.W. Compans, and D.W. Dixon.
Emory University, Atlanta, Ga.; Louisiana State University, Baton Rouge, La.; and Georgia State University, Atlanta, Ga., USA.

32. Development of a Dual-Reporter Assay for Identifying and Characterizing HIV-1 Tat Inhibitors.
P.A. Ward, R.G. Ptak, L.A. Pallansch, B.E. Beer, T.M, Fletcher III, M.G. Lewis, and J.A. Secrist III.
Southern Research Institute, Frederick, Md., and Birmingham, Ala., USA.

33. Suppression of HIV-1 Replication by an HIV-1 Dependent Anti-gene Expression Vector with the Cre-loxP System.
T. Nagawa, Y. Habu, N. Miyano-Kurosaki, and H. Takaku.
Chiba Institute of Technology, Chiba, Japan.

34. Inhibition of Human Immunodeficiency Virus Type 1 (HIV-1) by Ribozyme Expression Baculovirus Vector.
H. Kaneko, T. Abe, N. Miyano-Kurosaki, and H. Takaku.
Chiba Institute of Technology, Chiba, Japan.

35. Mechanism of 2',3'-Dideoxyinosine's Drug Interaction with Allopurinol, Ganciclovir and Tenofovir Disoproxil Fumarate.
A.S. Ray, A. Mahmoudi, and A. Fridland.
Gilead Sciences, Foster City, Calif., USA.

36. In Vitro Synergy Between the CD4 Down-Modulating Compound, CADA, and Multiple Anti-HIV Drugs.
K. Vermeire, S. Hatse, E. De Clercq, K. Dey, M.F. Samala, A. Sodoma, T.W. Bell, and D. Schols.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; and University of Nevada, Reno, Nevada, USA.

37. An Approach to Development of Anti-HIV Agents with Double Antiviral Protection.
A.V. Serbin, O.L. Alikhanova, I.V. Timofeev, N.G. Perminova, M.Y. Bourstain, and A.G. Bukrinskaya.
Health RDF, Moscow; SRC VB "Vektor", Koltsovo; and Ivanovsky Institute of Virology, Moscow, Russia.

38. Induction of Cellular Resistance to HIV-1 Nucleoside Reverse Transcriptase Inhibitors by Breast Cancer Resistance Protein (BCRP).
X. Wang, T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama, and M. Baba.
Kagoshima University, Kagoshima; and Japanese Foundation for Cancer Research, Tokyo, Japan.

39. Pre-Steady State Kinetic Analysis of the Incorporation of FTC-5'TP and 3TC 5'-triphosphate by Mutant HIV-1 RTs K65R, K65R/Q151M and Q151M.
G.B. Mulamba, L. Rimsky, F. Myrick, K. Borroto-Esoda, and J.Y. Feng.
Triangle Pharmaceuticals, Inc., Durham, N.C., USA.

40. Molecular Mechanism Studies of Tenofovir Against AZT and 3TC Drug Resistant Mutants.
Y. Chong, N. Akula, and C.K. Chu.
University of Georgia, Athens, Ga., USA.

41. Development of TK1 Deficiency Resulting in Cellular Resistance Against AZT in Human T-Lymphoid Cells is Prevented by Inhibition of the DNA Methyltransferases.
G. Hoever, B. Groeschel, H.W. Doerr, and J. Cinatl Jr.
Johann Wolfgang Goethe University Frankfurt, Frankfurt, Germany.

42. Effects of HIV Protease Inhibitors (PIs) on Lipid Accumulation and Glucose Uptake in Human Adipocytes.
G. Bahador, G.-X. He, and T. Cihlar.
Gilead Sciences, Foster City, Calif., USA.

43. Effect of Mycophenolic Acid on Intracellular Dioxolane Guanosine Triphosphate Levels in PBMC.
F. Myrick, R. St. Claire, R. Hart, and K. Borroto-Esoda.
Triangle Pharmaceuticals, Inc., Durham, N.C., USA.

44. The Antiviral Activity of the CXCR4 Antagonist AMD3100 is Independent of the Cytokine-Induced CXCR4/HIV Coreceptor Expression Level.
K. Princen, S. Hatse, K. Vermeire, G.J. Bridger, R.T. Skerlj, E. De Clercq, and D. Schols.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; and AnorMED, Inc., Langley, B.C., Canada.

45. Development of a CCR5-tropic HIV-1 Transmission Inhibition Assay for Testing Topical Microbicides.
C. Osterling, C. Lackman-Smith, N. Thompson, R. Ptak, O. Hartley, M. Lewis, and B.E. Beer.
Southern Research Institute, Frederick, Md., USA; and Centre Medical Universitaire, Geneva, Switzerland.

46. The Humanized Anti-CCR5 Antibody PRO 140 Effectively Inhibits HIV-1 Entry Without Inhibiting RANTES-Induced Calcium Mobilization.
B. O'Hara, J.P. Gardner, T.J. Ketas, B.M. Sullivan, S.I. Rosenfield, K.A. Nagashima, P.J. Maddon, and W.C. Olson.
Progenics Pharmaceuticals, Inc., Tarrytown, N.Y., USA

47. Studies of the Intracellular Metabolism of Nucleoside Amino Acid Phosphoramidates utilizing 31P NMR and LC/MS.
J. Kim and C.R. Wagner.
University of Minnesota, Minneapolis, Minn., USA.

48. Decreased Expression of Deoxycytidine Kinase (dCK) in Lymphocytes of Human Immunodeficiency Virus Type 1 (HIV-1) Infected Patients Under Highly Active Antiretroviral Therapy (HAART) with a Detectable Virus Load.
B. Morgenstern, H.W. Doerr, and J. Cinatl Jr.
Johann Wolfgang Goethe University Frankfurt, Frankfurt, Germany.

49. The Tat Antagonist Neomycin B Hexa-Arginine Conjugate Inhibits gp120-Induced Death of Human Neuroblastoma Cells.
A. Lapidot, M.V. Catani, M.T. Corasaniti, M. Ranalli, D. Amantea, A. Litovchick, and G. Melino.
Weizmann Institute of Science, Rehovot, Israel; University of Rome Tor Vergata, Rome; and University of Catanzaro “Magna Graecia”, Italy.

50. A Short and Efficient Route Towards Enantiomerically Pure Carbocyclic Analogues of Thymidine: New Substrates for Thymidylate Kinase.
O.R. Ludek, J. Balzarini, E. De Clercq, and C. Meier.
University of Hamburg, Hamburg, Germany; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

51. Interaction of Cholinesterases with CycloSal Nucleotides.
C. Ducho, J. Balzarini, and C. Meier.
University of Hamburg, Hamburg, Germany; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

52. A Publicly Available Database for Compounds Tested Against HIV and Other Viral Pathogens.
L.N. Callahan, L.W. Cooney, D.F. Metzler, D.F. Rezvani, J. Zhang. B. Laughon, C. Tseng, and M. Nasr.
Cygnus Corp., Inc., Rockville; and NIH, NIAID, Bethesda, Md., USA.

53. Phosphoramidate Derivatives of Carbocyclic L-d4A Nucleosides and Related Analogues.
S. Daluge, K. Gudmundsson, Z. Wang, E. McLean, L. Condreay, L. Johnson, R. Hazen, S. Srinivasan, A. Hassan-Abdallah, Y. Wang, R. Pathirana, A. Siddiqui, C. McGuigan, E. De Clercq, and J. Balzarini.
GlaxoSmithKline, Research Triangle Park, N.C., USA; Cardiff University, Cardiff, U.K.; and Rega institute for Medical Research, K.U. Leuven, Leuven, Belgium.

54. Unique Mechanism of L-FMAU against Hepatitis B Virus: A Molecular Dynamics Study.
Y. Chong and C.K. Chu.
The University of Georgia, Athens, Ga., USA.

55. Dinucleotides as Novel Class of Anti-Hepatitis B Virus Agents: Evaluation of ORI-9020 in a Transgenic Mouse Model.
J.G. Julander, R.P. Iyer, A. Roland, Y. Jin, S. Mounir, and J.D. Morrey.
Utah State University, Logan, Utah; and Spring Bank Technologies, Inc., Worcester, Mass., USA.

56. Imino Sugars with Greatly Enhanced Anti-Hepatitis B and BVDV Activity and Less Toxicity than DNJ.
A. Mehta, S. Ouzounov, R. Jordan, E. Simsek, X. Lu, R.M. Moriarty, G. Jacob, R.A. Dwek, and T.M. Block.
Thomas Jefferson University, Doylestown, Pa.; University of Illinois at Chicago, Chicago, Ill.; Synergy Pharmaceuticals, Inc., Somerset, N.J., USA; and University of Oxford, Oxford, U.K.

57. SeqHepB: A Hepatitis B Virus Sequence Analysis Program for Identifying HBV Mutants Associated with Antiviral Resistance Including Lamivudine, HBIG, Famciclovir, and Adefovir.
A. Bartholomeusz, S.A. Locarnini, A. Ayres, W. Sievert, J. Sasadeusz, P. Angus, and Members of the Collaborative Consortium.
Victorian Infectious Diseases Reference Laboratory, North Melbourne; Monash Medical Center, Clayton; Royal Melbourne Hospital, North Melbourne; and Austin Medical Repatriation Center, Heidelberg, Australia.

58. Ultrastructural Study of HBV-Producing Cell Lines Cultured in the Absence or Presence of Selective Inhibitors of HBV Replication.
C. Ying, A. Van Lommel, M. Van Ranst, E. De Clercq, and J. Neyts.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

59. LB80317, a Novel Anti-HBV Agent, is Very Potent Against Wild Type as well as Lamivudine-Resistant HBV Viruses.
J. Kim, J.-R. Choi, Y.Z. Kim, W.Y. Cho, Y.G. Cho, G.W. Kim, M.K. Lee, T.G. Kim, J.-T. Hwang, D.G. Cho, K.Y. Roh.
LG Life Sciences, Daejeon, Republic of Korea.

166. Collaboration with the National Cancer Institute (NCI) in the Development of Antiviral Therapeutics.
B. Gabrielsen.
NCI-Frederick, Frederick, Md., USA.

Tuesday, April 29

Oral Session III: Herpesviruses

Chairpersons: David I. Bernstein, James J. McSharry

8:30am Plenary Speaker – Prusoff Award
Johan Neyts, Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium
"Novel Strategies for Treatment of Infections with Herpes, Flavi, Entero, and Poxviruses"

9:00 60. A Thymidine Phosphorylase-Stable Analog of BVDU with Significant Antiviral Activity.
V. Nair, S. Guenther, J. Balzarini, and E. De Clercq.
University of Georgia, Athens, Ga., USA; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

9:15 61. Discovery of a New Family of Bicyclic Dideoxy Nucleosides Which Inhibit HCMV by a Non-Nucleoside Mechanism.
C. McGuigan, R.N. Pathirana, E. De Clercq, and J. Balzarini.
Cardiff University, Cardiff, U.K.; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

9:30 62. In Vitro Anti-Herpesvirus Activity of 4-Hydroxyquinoline Carboxamide (4-HQC) Analogs, A Novel Class of Non-Nucleoside Broad-Spectrum Polymerase Inhibitors.
E.R. Kern, C.B. Hartline, E.A. Harden, S.L. Williams, N.L. Kushner, and R.J. Brideau.
The University of Alabama School of Medicine, Birmingham, Ala; and Pharmacia Infectious Disease Research, Kalamazoo, Mich., USA.

9:45 63. The Antiviral Activity of CTC-8 a Member of Novel Class of Herpesvirus Selective Inhibitors Based on Prostaglandin Analogues with a Putative Cellular Target for the Inhibition of Herpes Viruses.
T.J. Fitzmaurice, D.R. Harper, and H.J. Field.
Centre for Veterinary Science, Cambridge; and Charterhouse Therapeutics, Ltd., Oxford, U.K.

10:00 Break

10:30 64. The IκB Kinase IKK is the Molecular Target for Anti-Herpetic Cyclopentenones.
C. Amici, G. Belardo, A. Ciucci, C. Volpi, T. Guyot, P. Evans, A. Happe, T. Snape, S. Roberts, and M.G. Santoro.
University of Rome Tor Vergata, Rome, Italy; Charterhouse Therapeutics, Ltd., Oxford; and University of Liverpool, Liverpool, U.K.

10:45 65. Evaluation of a Recombinant Live Attenuated Herpes Simplex Virus Type 2 Vaccine in Guinea Pigs.
M.N. Prichard, R. Kaiwar, W.T. Jackman, E.R. Kern, D.C. Quenelle, D.J. Collins, B.P. Herrod, K.M. Gustin, G.M. Kemble, and R.R. Spaete.
MedImmune Vaccines, Inc., Mountain View, Calif.; and University of Alabama School of Medicine, Birmingham, Ala., USA.

11:00 66. A New Antiviral Mechanism: Mutation of Human Cytomegalovirus Gene UL27 Confers Resistance to the Antiviral Drug 1263W94.
G. Komazin, R.G. Ptak, B.T. Emmer, D.L. Evers, L.B. Townsend, and J.C. Drach.
University of Michigan, Ann Arbor, Mich., USA.

11:15 67. CMV423, A Potent and Selective Inhibitor of Human Herpesvirus 6 Exerts its Antiviral Action Through Inhibition of Cellular Protein Tyrosine Kinase Activity.
L. De Bolle, G. Andrei, R. Snoeck, A. Van Lommel, M. Otto, A. Bousseau, C. Roy, E. De Clercq, and L. Naesens.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; Pharmasset, Tucker, Ga., USA; and Aventis Pharma, Paris, France.

11:30 Plenary Speaker
Ken Sibley, Myers Bigel Sibley & Sajovec, P.A, Raleigh, N.C., USA
"Scientific Collaboration, Patent Law and Antiviral Research"

12:00 Lunch

Tuesday, April 29

Oral Session IV: Minisymposium – Perspectives in Biodefense, How Prepared Are We?

Chairpersons: Earl R. Kern, Catherine A. Laughlin

1:30pm	Industry - Gail Cassell, Lilly Research Laboratories, Indianapolis, Ind., USA
1:50	National Institutes of Health – James Meegan, Bethesda, Md., USA
2:10	Centers for Disease Control – James LeDuc, Atlanta, Ga., USA
2:30	USAMRIID – Peter Jahrling, Frederick, Md., USA
2:50	Food and Drug Administration – Lewis Schrager, Rockville, Md., USA.
3:10	Panel Discussion Moderator, Catherine A. Laughlin

Tuesday, April 29

Poster Session II: Herpesviruses, Poxviruses

4 p.m. – 6 p.m.

68. Inhibition of Herpesvirus Replication In Vitro by a Series of 4-Oxo-Hydroquinoline Carboxamides with Viral Polymerase Activity.
C.B. Hartline, E.A. Harden, S.L. Williams, N.L. Kushner, R.J. Brideau, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala; and Pharmacia Infectious Disease Research, Kalamazoo, Mich., USA.

69. In Vitro Activity of Alkoxyalkyl Esters of Cidofovir Against Replication of Herpesviruses.
S.L. Williams, C.B. Hartline, N.L. Kushner, E.A. Harden, J.R. Beadle, K.Y. Hostetler, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala; and VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA.

70. Bicyclic Furo Pyrimidine Nucleosides with Haloaryl Side Chains.
A. Angell, C. McGuigan, A. Jukes, H. Baruki, S. Blewett, G. Andrei, R. Snoeck, E. De Clercq, and J. Balzarini.
Cardiff University, Cardiff, U.K.; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

71. Combined Anti HSV-1 Activity of New Dimeric Derivatives of Netropsin with Modified Nucleosides and Phosphonoacetic Acid (PAA) in Vero Cells.
G. Galegov, V. Andronova, S. Grokhovsky, A. Surovaya, N. Leontieva, and G. Gursky.
The D.I. Ivanovsky Institute of Virology, Moscow; V.A. Engelhgardt Institute of Molecular Biology, Moscow, Russia.

72. Antiherpetic Activity of Some 5-arylethynyl Derivatives of 2'-deoxyuridine in Vero Cells.
V. Andronova, M. Skorobogaty, Yu. Berlin, V. Korshun, and A. Galegov.
The D.I. Ivanovsky Institute of Virology, Moscow; and The Shemiakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia.

73. Dynamics of N-Methanocarbathymidine Antiherpetic Activity.
M. Huleihel, L. Zalah, M. Talyshinsky, V.E. Marquez, and R. Agbaria.
Ben-Gurion University of the Negev, Beer-Sheva, Israel; and National Cancer Institute at Frederick, National Institutes of Health, Frederick, Md., USA.

74. Antiviral Activity of Triplex-Forming Oligonucleotides Against HSV-1.
J.D. Kriesel, B.B. Jones, and S.L. Spruance.
University of Utah School of Medicine, Salt Lake City, Utah, USA.

75. Metalloporphyrins as Agents Against HSV.
D.W. Dixon, A. Alam, L. Hodge, L.G. Marzilli, and A. Sears.
Georgia State University, Atlanta, Ga.; Louisiana State University, Baton Rouge, La.; and Tampa Bay Research Institute, St. Petersburg, Fla., USA.

76. Specific Inhibition of Virus Replication by Binding of a N,N'-bisheteryl Derivative of Dispirotripiperazine (DSTP) to Heparin Sulfate (HS) Residues on the Cell Surface.
M. Schmidtke, A. Karger, A. Meerbach, R. Egerer, A. Stelzner, and V. Makarov.
Friedrich Schiller University, Jena; Federal Research Center for Virus Diseases of Animals, Insel Riems, Germany; and State Scientific Center of the Russian Federation, "NIOPIK", Moscow, Russia.

77. The Synergistic Effects of Betulin with Acyclovir Against Herpes Simplex Viruses.
Y. Gong, C. Luscombe, I. Gadawski, D. Cheung, T. Tam, and S. Sacks.
Viridae Clinical Sciences, Inc., Vancouver, B.C.; Canada; NaturTek, Duluth, Minn., USA; and The University of British Columbia, Vancouver, B.C., Canada.

78. Establishment of Herpes Simplex Virus (HSV) Latency Model Using PC12 Cells and the Antiviral Activity of Pencyclovir on HSV Reactivation.
Y. Gong, J. Meligeni, Y.-H. Su, T. Tam, I. Gadawski, and S. Sacks.
Viridae Clinical Sciences, Inc., Vancouver, Canada; Novartis Pharmaceuticals Corporation, East Hanover, N.J, USA; Jefferson Center for Biomedical Research, Doylestown, Pa., USA; and the University of British Columbia, Vancouver, Canada.

79. Construction of an Expression Vector Encoding Glycoprotein D of Herpes Simplex Virus Type-1 for Detection of the Expressed Protein by Fluorescent Technique.
H. Soleimanjahi, M.H. Roostaee, F. Mahboodee, M.J. Rassaee, and T. Bamdad.
Tarbiat Modarres University; and Pasteur Institute, Tehran, Iran.

80. Construction of an Expression Vector Containing Main Neutralizing Epitopes of Herpes Simplex Virus Type 1 Glycoprotein B and its Expression in Mammalian Cell Lines.
T. Bamdad, M.H. Roostaee, M. Sadegheezadeh, and F. Mahboodee.
Tarbiat Modarres University; and Pasteur Institute, Tehran, Iran.

81. Application of Recombinant Glycoprotein G and D (gG-1 and gD-1) of Herpes Simplex Virus Type 1 (HSV-1) for Detection of Specific Antibodies.
M.H. Roostaee, K. Zandee, H. Soleimanjahee, and Z. Meshkat.
Tarbiat Modarres University, Tehran, Iran.

82. Molecular Analysis of Clinical Isolates of ACV-Resistant Herpes Simplex Virus.
D. Chibo, J. Druce, L. Buchanan, J. Sasadeusz, and C. Birch.
Victorian Infectious Diseases Reference Laboratory; and Royal Melbourne Hospital, North Melbourne, Victoria, Australia.

83. Intracellular metabolism of Acyclovir and Deoxythymidine in Cell Culture System Using HSV-1 and its Acyclovir-Resistant Mutant Strains.
P.K. Bae, J.H. Kim, H.S. Kim, S.-G. Paik, I.K. Chung, and C.-K. Lee.
Korea Research Institute of Chemical Technology, Taejon; Chungnam National University, Taejon; and Yonsei University, Seoul, Korea.

84. New Quantitative Method of In-Line Description of Virus-Cell Interaction at Every Stage.
A.O. Fedchuk, A.S. Fedchuk, L.I. Shitikova, and O.P. Fedchuk.
I.I. Mechnikov National University; and Ukrainian Research Anti-Plague Institute, Odesa, Ukraine.

85. An Immunomodulating Peptide (CEL-1000) Elicits Protection Against HSV-1.
K.S. Rosenthal, N. Goel, and D.H. Zimmerman.
Northeastern Ohio Universities College of Medicine, Rootstown, Ohio; and Cel-Sci Corporation, Vienna, Va., USA.

86. In Vivo Anti-Herpes Simplex Virus Activity of Resveratrol, A Cyclin Dependent Kinase Gene Inhibitor.
J. Docherty, M.M. Fu, T. Stoner, J. Smith, M. Lesniewski, C.-C. Tsai, and T. Booth.
Northeastern Ohio Universities College of Medicine, Rootstown, Ohio; Kent State University, Kent, Ohio, USA; and Royalmount Pharmaceuticals, Inc., Montreal, Canada.

87. In Vivo and In Vitro Antiherpetic Effects of Hydroxytolans.
C.C. Tsai, M.L. Lesniewski, B. Liu, R.R. Parakulam, M.M. Fu, and J.J. Docherty.
Kent State University, Kent; and Northeastern Ohio Universities College of Medicine, Rootstown, Ohio, USA.

88. Sulfonated Halogenated Porphyrins have Potent Virucidal Activity Against Herpes Simplex Viruses 1 and 2 In Vitro and In Vivo.
A.E. Sears, D.W. Dixon, K. Gould, L.K. Hodge, and L. Marzilli.
Tampa Bay Research Institute, St. Petersburg, Fla.; Georgia State University; Atlanta; and Yerkes Regional Primate Center and Emory University, Atlanta, Ga., USA.

89. Evaluation of Formulated Dendrimer SPL 7013 as a Microbicide.
D.I. Bernstein, N. Bourne, N.K. Ayisi, J. Ireland, B. Matthews, T. McCarthy, and S. Sacks.
Children's Hospital Medical Center, Cincinnati, Ohio; Viridae Clinical Sciences, Inc., Cincinnati, Ohio, USA; and Starpharma, Ltd., Parkville, Australia.

90. Abreva (10% n-docosanol cream) vs 5% Acyclovir (ACV) in 80% Dimethyl Sulfoxide (DMSO) in the Treatment of Experimental Dorsal Cutaneous Herpes Simplex Virus 1 (HSV-1) Infection in the Hairless Guinea Pig.
M. McKeough and S. Spruance.
University of Utah, Salt Lake City, Utah, USA.

91. Antiviral Activity of CycloSaligenyl Prodrugs of BVDU Against Herpesviruses (HSV, VZV, HCMV, EBV).
A. Meerbach, C. Meier, H.-M. Meckel, A. Lomp, and P. Wutzler.
Friedrich-Schiller University, Jena, Germany.

92. Interaction of the Bicyclic Nucleoside Analogues (BCNAs) with Human Varicella-Zoster Virus (VZV) and Simian Varicella Virus (SVV) Thymidine Kinase and VZV DNA Polymerase.
R. Sienaert, L. Naesens, G. Luoni, M. Wathen, W. Gray, G. Andrei, R. Snoeck, E. De Clercq, C. McGuigan, and J. Balzarini.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium; Cardiff University, Cardiff, U.K.; Pharmacia Corp., Kalamazoo, Mich.; and College of Medicine, West Markham, Arkansas, USA.

93. Potent Antiviral Activity of Red Microalgal Polysaccharide Against Varicella Zoster Virus.
M. Huleihel, V. Erukhimovitch, M. Talyshinskhy, Y. Souprun, and S.M. Arad.
Ben-Gurion University of the Negev, Beer-Sheva, Israel.

94. Penetration of Highly Active Anti-VZV Nucleoside Analogues Into and Through Full-Thickness Skin.
C.A. Jarvis, C. McGuigan, and C.M. Heard.
Cardiff University, Cardiff, U.K.

95. Cell Death Induced by (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) in Varicella Zoster Virus Thymidine Kinase Expressing Cells: Activation of c-Jun/AP-1 and FasL/caspase-8.
M.T. Tomicic-Christmann, C. Friedrichs, M. Christmann, P. Wutzler, R. Thust, and B. Kaina.
University of Mainz, Mainz; and University of Jena, Jena, Germany.

96. Varicella Zoster Virus Infection Induces Cyclin Dependent Kinase 2/Cyclin E Expression and Replication is Blocked by Roscovitine, a cdk2 Inhibitor.
J.F. Moffat, S.L. Taylor, S. Leisenfelder, and P.R. Kinchington.
SUNY Upstate Medical University, Syracuse, N.Y., USA.

97. Antiviral Structure-Activity Relationships of the First and Second Generation Methylenecyclopropane Nucleoside Analogues.
J. Zemlicka, E.R. Kern, Y.-C. Cheng, and J.C. Drach.
Wayne State University School of Medicine, Detroit, Mich.; University of Alabama School of Medicine, Birmingham, Ala.; Yale University School of Medicine, New Haven, Conn.; and University of Michigan, Ann Arbor, Mich., USA.

98. In Vitro Activity of Methylenecyclopropane Analogs of Nucleosides Against Herpesvirus Replication.
N.L. Kushner, S.L. Williams, C.B. Hartline, E.A. Harden, X. Chen, S. Zhou, J. Zemlicka, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala; and Wayne State University School of Medicine, Detroit, Mich., USA.

99. Synthesis and Biological Evaluation of Bicyclic Furano Pyrimidine Nucleosides as Inhibitors of Human Cytomegalovirus.
M.R. Kelleher, O. Bidet, A. Carangio, C. McGuigan, H. Weldon, G. Andrei, R. Snoeck, E. De Clercq, and J. Balzarini.
Cardiff University, Cardiff, U.K.; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

100. Synthesis and Biological Evaluation of a New Series of 5-Alkylaryl Nucleoside Analogues Showing Anti-HCMV Activity.
G. Luoni, C. McGuigan, G. Andrei, R. Snoeck, E. De Clercq, and J. Balzarini.
Cardiff University, Cardiff, U.K.; and Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

101. Alkoxyalkyl Esters of Adefovir: Antiviral Activity Against Cytomegalovirus and HIV-1, In Vitro.
K.A. Aldern, J.R. Beadle, S.M. Rostami, and K.Y. Hostetler.
VA San Diego Healthcare System; and University of California, San Diego, La Jolla, Calif., USA.

102. Synthesis of Alkoxyalkyl Esters of (R)- and (S)-HPMPA and Antiviral Activity Against Herpesviruses, In Vitro.
S.L. Ciesla, W.B. Wan, K.A. Aldern, J.R. Beadle, and K.Y. Hostetler.
VA San Diego Healthcare System, and University of California, San Diego, La Jolla, Calif., USA.

103. Anticonvulsive Drug Valproic Acid Increases Replication of HCMV in Human Foreskin Fibroblasts and Retinal Pigment Epithelial Cells in Therapeutic Concentrations.
M. Michaelis, A. Reinisch, N. Köhler, H.W. Doerr, H. Nau, and J. Cinatl Jr.
Johann Wolfgang Goethe University Frankfurt, Frankfurt; Zentrum fur Lebensmitelwissenschaften, Bischofsholer Damm, Hannover, Germany.

104. In Vivo Activity of 1-O-Hexadecyloxypropyl-cidofovir (HDP-CDV) Against Murine and Human Cytomegalovirus Infections.
D.J. Bidanset, D.C. Quenelle, D.J. Collins, B.P. Herrod, J.R. Beadle, K.Y. Hostetler, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala; and VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA.

105. BAY 38-4766: An Effective Antiviral Agent Against Guinea Pig Cytomegalovirus (GPCMV).
M. Schleiss, S. Hallenberger, K. Henninger, F.J. Bravo, G. Stroup, and D.I. Bernstein.
Children's Hospital Medical Center, Cincinnati, Ohio; and Bayer Corporation, Cambridge, Mass., USA.

106. Effective Treatment of Congenital Cytomegalovirus (CMV) by Cyclic HPMPC using the Guinea Pig Model of Congenital CMV.
D.I. Bernstein, F. Bravo, and M.R. Schleiss.
Children's Hospital Medical Center, Cincinnati, Ohio, USA.

107. Characteristics of Epstein-Barr Virus Encoded Protein Kinase.
E. Gershburg, K. Hong, and J.S. Pagano.
University of North Carolina at Chapel Hill, Chapel Hill, N.C., USA.

108. Studying of AntiEpstein-Barr Virus Activity of 6-Azacytidine and its Acyclic Derivative.
N. Nesterova, N. Dyachenko, I. Alexeeva, S. Zagorodnaya, and G. Baranova.
National Academy of Sciences of Ukraine, Kyiv, Ukraine.

109. Cytogenetic Genotoxicity of New CycloSaligenyl Prodrugs of Ganciclovir and Aciclovir.
C. Friedrichs, R. Thust, C. Meier, and P. Wutzler.
Friedrich Schiller University, Jena; and University of Hamburg, Germany.

110. Inhibitory Effect of Alkoxyalkyl Esters of Acyclic Nucleoside Phosphonates Against Orthopoxvirus Replication.
K.A. Keith, W.B. Wan, S.L. Ciesla, J.R. Beadle, K.Y. Hostetler, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala.; VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA

111. Effects of 9-[2-(Phosphonomethoxy)ethyl]guanine (PMEG), Hexadecyloxypropyl-PMEG and Octadecyloxyethyl-PMEG on Replication of HIV-1, Herpesviruses and Poxviruses, In Vitro.
N. Valiaeva, K.A. Keith, K.A. Aldern, J.R. Beadle, E.R. Kern, and K.Y. Hostetler.
VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif.; and The University of Alabama School of Medicine, Birmingham, Ala., USA.

112. Antiviral Activity of Cyclopentenyl Nucleosides Against Orthopox (Smallpox, Monkeypox, and Cowpox), West Nile and Ebola Viruses.
Y.H. Jin, R.O. Baker, J. Huggins, and C.K. Chu.
The University of Georgia, Athens, Ga.; and USAMRIID, Ft. Detrick, Md., USA.

113. Prevention of Poxvirus Infection by Tetrapyrroles.
A.R.M. Chen-Collins, D.W. Dixon, A.N. Vzorov, L.G. Marzilli, and R.W. Compans.
Emory University, Atlanta; Georgia State University; Atlanta, Ga.; and Louisiana State University, Baton Rouge, La., USA.

114. Efficacy of Single or Interval Dosing with Cidofovir Given Before or After Infection with Orthopoxviruses in Mice.
E.R. Kern, D.J. Collins, B.P. Herrod, and D.C. Quenelle.
The University of Alabama School of Medicine, Birmingham, Ala., USA.

115. Activity of Cidofovir in a Murine Model for Cutaneous Vaccinia Virus Infection.
J. Neyts and E. De Clercq.
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

116. Predictability of Genome Levels in Blood for Levels in Tissues of Cowpox Infected Mice.
S.H. Zwiers, J.D. Shamblin, R.O. Baker, and J.W. Huggins.
USAMRIID, Fort Detrick, Md., USA.

117. Combinatorial Antibodies Against Orthopoxviruses.
N. Tikunova, V. Morozova, E. Bovshik, E. Belanov, A. Guskov, T. Yun, A. Ilyichev, and L. Sandakhchiev.
State Research Center of Virology and Biotechnology VECTOR, Koltsovo, Novosibirsk Region, Russia.

118. Should the Guidelines for Revaccination Against Smallpox be Changed?
S. Baron, J. Pan, and J. Poast.
University of Texas Medical Branch, Galveston, Tex., USA

132. Oral Pharmacokinetics and Tissue Distribution of 1-0-Hexadecyloxypropyl1-[2-14C] Cyclic Cidofovir in Mice.
J. Trahan, S.L. Ciesla, K.L. Winegarden, and K.Y. Hostetler.
VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA.

Wednesday, April 30

Oral Session V: Hepatitis C Virus, Flaviviruses

Chairpersons: John D. Morrey, Johan Neyts

8:30am119. Structure Activity Relationship of 2' Modified Nucleosides for Inhibition of Hepatitis C Virus.
A.B. Eldrup, S. Bera, N. Bhat, J. Brooks, S.S. Carroll, P.D. Cook, P. Dande, M. MacCoss, D. MacMasters, D.B. Olsen, T.P. Prakash, M. Prhavc, Q. Song, J. Tomassini, J. Xia, and B. Bhat.
Isis Pharmaceuticals, Carlsbad, Calif.; Merck Research Laboratories, West Point, Pa.; and Merck Research Laboratories, Rahway, N.J., USA.

8:45 120. Synthesis and Pharmacokinetic Properties of Nucleoside Analogues as Possible Inhibitors of HCV RNA Replication.
B. Bhat, J. Xia, T.P. Prakash, P.D. Cook, D.B. Olsen, S.S. Carroll, J. Tomassini, M. MacCoss, and A.B. Eldrup.
Isis Pharmaceuticals, Carlsbad, Calif.; Merck Research Laboratories, West Point, Pa.; and Merck Research Laboratories, Rahway, N.J., USA.

9:00 121. 2'-Modified Nucleoside Analogs as Inhibitors of Hepatitis C RNA Replication.
D.B. Olsen, B. Bhat, M. Bosserman, S.S. Carroll, L. Colwell, R. De Francesco, A.B. Eldrup, O. Flores, K. Getty, R. LaFemina, M. MacCoss, G. Migliaccio, A.L. Simcoe, C.A. Rutkowski, M.W. Stahlhut, J.E. Tomassini, and B. Wolanski.
Merck Research Laboratories, West Point, Pa., and Rahway, N.J.; Isis Pharmaceuticals, Carlsbad, Calif., USA; and Istituto di Ricerche di Biologia Molecolare, Pomezia (Roma), Italy.

9:15 122. Enhancement of Activity Against HCV Replication by Combination Therapy in the HCV Replicon Model.
B. Korba, A. Ciancio, C. Okuse, and J. Rinaudo.
Georgetown University, Rockville, Md., USA; Ospedale S. Giovanni Battista di Torino, Italy; and St. Marianna University School of Medicine, Kawasaki, Japan.

9:30 123. A Cell-Based Assay for Evaluation of Anti-Viral Agents Against HCV.
A. Zauberman, S. Aviel, E. Ilan, G. Kitzis-Livneh, K. Shochat, O. Nussbaum, D. Landstein, R. Eren, and S. Dagan.
XTL Biopharmaceuticals, Ltd., Rehovot, Israel.

9:45 124. Discovery of a Novel Class of HCV NS5B RNA Dependent RNA Polymerase Inhibitors: SAR Studies and Activity in Replicon Cells.
L. Chan, T.J. Reddy, M. Proulx, S.K. Das, O. Pereira, M. Courchesne, C. Roy, C. Yannopoulos, C. Poisson, N. Nguyen-Ba, L. Halab, R. Bethell, M.-Q. Zhang, M. David, L.L'Heureux, J. Bédard, M. Hamel, and O. Nicolas.
Shire BioChem Inc., Laval, QC, Canada.

10:00 Break

10:30 125. The Trimera Model: A Tool for Differential Gene Expression Analysis in HCV-Infected Human Liver Cells.
A. Zauberman, S. Banin, E. Ilan, N. Kaminski, and S. Dagan.
XTL Biopharmaceuticals, Ltd., Rehovot; and Sheba Medical Center, Tel Hashomer, Israel.

10:45 126. Evaluation of Therpeutics Against West Nile Virus in a Hamster Model.
C.W. Day, J.D. Morrey, and R.W. Sidwell.
Utah State University, Logan, Utah, USA.

11:00 Invitation to 17th ICAR

11:15 ISAR Business Meeting

12:00 Lunch

Wednesday, April 30

Oral Session VI: Poxviruses

Chairpersons: Erik De Clercq, Walla L. Dempsey

1:30pm Plenary Speaker
Walla L. Dempsey, NIH, NIAID, Bethesda, Md., USA
"What are the issues in smallpox vaccination?"

2:00 127. Cidofovir Treatment of Variola (Smallpox) in the Hemorrhagic Smallpox Primate Model and the IV Monkeypox Primate Model.
J.W. Huggins, S.H. Zwiers, R.O. Baker, L.E. Hensley, T. Larsen, M.J. Martinez, and P.B. Jahrling.
USAMRIID, Fort Detrick, Md., USA.

2:15 128. Aerosolized Cidofovir is Retained in the Lung and Protects Mice Against Lethal Intranasal Cowpox Virus Infection.
C.J. Roy, R. Baker, K. Washburn, and M. Bray.
USAMRIID, Fort Detrick, Md.; Stillmeadow Corporation, Sugarland, Tex.; and OCR/NIAID/NIH, Bethesda, Md., USA.

2:30 129. Vaccinia Skin Lesions in Immunosuppressed Hairless Mice can be Treated Topically but not Parenterally with Cidofovir.
D.F. Smee, K.W. Bailey, and R.W. Sidwell.
Utah State University, Logan, Utah, USA.

2:45 130. Effect of Oral Treatment with Alkoxyalkyl Esters of Cidofovir on Cowpox or Vaccinia Virus Infections in Mice.
D.C. Quenelle, D.J. Collins, B.P. Herrod, J.R. Beadle, W.B. Wan, K.Y. Hostetler, and E.R. Kern.
The University of Alabama School of Medicine, Birmingham, Ala.; The San Diego VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA.

3:00 131. Effect of Oral Ether Lipid Analogs of Cidofovir on Mortality and Viral Infectivity Levels in Tissues in a Lethal Ectromelia Virus Challenge Model.
R.M.L. Buller, G. Owens, J. Schriewer, J.R. Beadle, and K.Y. Hostetler.
St. Louis University Health Sciences Center, St. Louis, MO; and VA San Diego Healthcare System and University of California, San Diego, La Jolla, Calif., USA.

3:15 133. Development of a Rodent Model for Lethal Monkeypox Infections and its use in Antiviral Drug Testing.
R.O. Baker, R. Herrera, J. Shamblin, C. Hartmann, E. Mucker, S. Zwiers, and J.W. Huggins.
USAMRIID, Fort Detrick, Frederick, Md., USA.

3:30 134. Smallpox Model: Protection by IFN and Poly 1:CLC Despite Evasive Mechanisms.
S. Baron, A. Salazar, S. Pestka, J. Poast, and B. Clark.
University of Texas Medical Branch, Galveston, Tex.; Ribopharm, Inc., Washington, D.C.; PBL Biomedical Laboratories, Piscataway, N.J.; and Robert Wood Johnson Medical School-UMDNJ, Piscataway, N.J., USA.

3:45 Late Breaker Presentation

Wednesday, April 30

Poster Session III: Respiratory Viruses, Flaviviruses, and Other Viruses

4 p.m. – 6 p.m.

135. Inhibition of Clinically Important Respiratory Viruses by Beta-D-N^4-hydroxycytidine (NHC).
D.L. Barnard, V.D. Stowell, D.F. Smee, R.W. Sidwell, K. Watanabe, L.J. Stuyver, and M.J. Otto.
Utah State University, Logan, Utah; and Pharmasset, Inc., Tucker, Ga., USA.

136. Structural Analysis of the Activation of Ribavirin Analogs by NDP Kinase: Comparison with Other Ribavirin Targets.
D. Deville-Bonne, S. Gallois-Montbrun, Y. Chen, H. Dutartre, L. Mulard, S. Morera, J. Janin, M. Véron, and B. Canard.
Institut Pasteur, Paris; LEBS, CNRS; and ESIL-CNRS-AFMB, Marseille, France.

137. Anti-Influenza and Anti-Herpetic Activity of Decametoxin.
A.S. Fedchuk, V.P. Lozitsky, T.L. Gridina, L.I. Shitikova, and G.K. Palyi.
Ukrainian Research Anti-Plague Institute, Odesa; and N.I. Pyrogov Vinnitsa State Medical University, Vinnitsa, Ukraine.

138. Biologically Active Constituents of a Polyphenol Extract from Geranium Sanguineum L. with Anti-Influenza Virus Activity.
J. Serkedjieva, and S. Ivancheva.
Bulgarian Academy of Sciences, Sofia, Bulgaria.

139. Protease-Inhibitory Activity of a Plant Preparation with Anti-Influenza Virus Effect.
J. Serkedjieva, S. Antonova-Nikolova, I. Ivanova, S. Ivancheva, and R. Tzvetkova.
Bulgarian Academy of Sciences, Sofia University, Sofia, Bulgaria.

140. Effect of Cranberry Juice Constituents on Hemagglutination and Infectivity of Influenza Virus.
M. Schlesinger, E.I. Weiss, N. Hochman, I. Ofek, and Z. Zakay-Rones.
Hadassah-Hebrew University, Jerusalem; and Tel-Aviv University, Tel-Aviv, Israel.

141. Elaboration and Usage of 4D-QSAR Approach for Successful Antiviral Development.
V. Lozitsky, V. Kuz'min, A. Artemenko, R. Lozytska, A. Fedchuk, Yu. Boschenko, T. Gridina, L. Shitikova, L. Mudrik, J.J. Vanden Eynde, E. Muratov, and D. Kryzhanovsky.
Ukrainian Research Anti-Plague Institute, Odessa; National University, Odessa; Physico-Chemical Institute, Odessa, Ukraine; and University of Mons-Hainaut, Mons, Belgium.

142. Influenza Infection of the Embryonated Hen's Egg - An Alternative Model for In Vivo Evaluation of Antiviral Compounds.
A. Sauerbrei, A. Härtl, and P. Wutzler.
Friedrich-Schiller University; and Hans-Knöll Institute for Natural Products Research, Jena, Germany.

143. Transdermal Delivery Efficacy of Rimantadine Under Experimental Influenza Model in Mice.
I. Kravchenko, V. Lozitsky, R. Lozytska, and V. Larionov.
Odessa National University; Ukrainian I.I. Mechnikov Research Anti-Plague Institute; and AV Bogatsky Physics-Chemical Institute of NAS of Ukraine, Odessa, Ukraine.

144. Isolation of Disoxaril-Dependent Mutant of Coxsackievirus B1.
I. Nikolova and A.S. Galabov.
Bulgarian Academy of Sciences, Sofia, Bulgaria.

145. Clinical Coxsackievirus B3 (CVB3) Isolates Differ in Neutralization Pattern, Cell Tropism, and Pleconaril Sensitivity.
M. Schmidtke, E. Hammerschmidt, and V. Makarov.
Friedrich Schiller University, Jena, Germany; State Scientific Center of the Russian Federation "NIOPIK", Moscow, Russia.

146. Inhibition of Coxsackie B3 Virus Infection In Vitro and in a Murine Model of Virus-Induced Myocarditis by 2-(3,4-Dichlorophenoxy)-5-Nitrobenzonitrile.
E. Padalko, E. Verbeken, J. Aerts, E. De Clercq, and J. Neyts.
Rega Institute for Medical Research, K.U. Leuven, Leuven; University Hospitals, Leuven, Belgium.

147. Effects of Ribavirin on Murine Respiratory Syncytial Virus Disease: Evidence for Immunomodulation.
G. Bolger, N. Lapeyre, N. Dansereau, K. Klosowski, T. Mewhort, G. Berry, and M. Liuzzi.
Boehringer Ingelheim Canada Limited, Laval; and St. Mary's Hospital, Montreal, Quebec, Canada.

148. Broad Spectrum Anti-RNA Virus Activities of Titanium or Vanadium Substituted Polyoxotangstates.
S. Shigeta, S. Mori, E. Kodama, and T. Yamase.
Fukushima Medical University, Fukushima; Kyoto University, Kyoto; and Tokyo Institute of Technology, Tokyo, Japan.

149. In Vitro Anti-Rhinovirus Activity of a Tetrameric Anti-ICAM-1 Antibody (CFY-196).
F. Hayden and C. Crump
University of Virginia, Charlottesville, Va., USA.

150. Discovery and SAR Studies of a Novel Class of HCV NS5B RNA-dependent RNA Polymerase Inhibitors.
N. Nguyen-Ba, S.K. Das, C. Poisson, T.J. Reddy, M. Proulx, O. Pereira, M. Courchesne, C. Roy, C. Yannapoulos, L. Halab, L. Chan, M.-Q. Zhang, R. Bethell, L.L'Heureux, M. David, M. Hamel, O. Nicolas, and J. Bédard.
Shire BioChem, Inc., Laval, QC, Canada.

151. The Validation of GBV-B as a Surrogate Model for HCV in the Drug Discovery Process.
H. Bright, P. Watts, T. Carroll, and R. Fenton.
GlaxoSmithKline Medicines Research Centre, Stevenage, Hertfordshire, U.K.

152. Transplantation of HCV-Infected Hepatocellular Carcinoma (HCC) Cell-Line in the HCV-Trimera Mouse System.
E. Ilan, A. Levy, A. Zauberman, Y. Arazi, O. Nussbaum, R. Eren, I. Lubin, O. Ben-Moshe, A. Kitchinsky, A. Litchi, G. Zamir, A. Eid, O. Jurim, E. Galun, and S. Dagan.
XTL Biopharmaceuticals, Rehovot; Hadassah University Hospital, Jerusalem; and Rambam Medical Center, Haifa, Israel.

153. Optimisation of Peptide Based Inhibitors of Full Length Hepatitis C Virus NS3 Protease.
A. Poliakov, A. Johansson, E. Åkerblom, G. Lindeberg, A. Hallberg, and U.H. Danielson.
Uppsala University, Uppsala, Sweden.

154. Synthesis and Evaluation of S-acyl-2-thioethyl (SATE) Esters of Modified Nucleoside 5'-Monophosphates as Inhibitors of Hepatitis C RNA Replication.
T.P. Prakash, A.B. Eldrup, D.B. Olsen, S.S. Carroll, M. MacCoss, J. Tomassini, T.M. Prhavc, P.D. Cook, and B. Bhat.
Isis Pharmaceuticals, Carlsbad, Calif.; Merck Research Laboratories, West Point, Pa.; and Merck Research Laboratories, Rahway, N.J., USA.

155. Dissection of the Replicative Cycle of the Bovine Viral Diarrhea Virus to Allow Detailed Time-of-Antiviral Drug Addition Studies.
J. Paeshuyse, E. De Clercq, and J. Neyts
Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium.

156. Synergistic Direct Interactions Between IFN and RBV Against both BVDV and YFV as Surrogate Models of HCV Replication.
V.E. Buckwold, J. Wei, M. Wenzel-Mathers, and J. Russell.
Southern Research Institute, Frederick, Md., USA.

157. Potent Inhibition of NTPases/Helicases of the West Nile Virus (WNV) and Other Flaviviridae by Ring-Expanded ("Fat") Nucleoside and Nucleotide Analogues.
R.S. Hosmane, N. Zhang, H.-M. Chen, V. Koch, H. Schmitz, C.-L. Liao, A.I. Fattom, R.B. Naso, and P. Borowski.
University of Maryland, Baltimore, Md., USA; Bernhard-Nocht-Institut fur Tropenmedizin, Hamburg, Germany; National Defense Medical Center, Taipei, Taiwan; and Nabi, Rockville, Md., USA.

158. Ribavirin Efficacy Against West Nile Virus After Multiple Passages in Cell Culture.
C.W. Day, J.D. Morrey, and R.W. Sidwell.
Utah State University, Logan, Utah, USA.

159. A Neutral Red Uptake Assay for the Rapid Screening of Antiviral Compounds Against the Filoviruses, Ebola and Marburg.
C.A. Whitehouse, D. Miller, M. Bray, and J. Paragas.
U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md., USA.

160. Human Miniantibodies Specific to Ebola Virus.
N. Tikunova, T. Batanova, and A. Chepurnov.
State Research Center of Virology and Biotechnology VECTOR, Koltsovo, Novosibirsk Region, Russia.

161. Antiviral Activity of new Thiosemicarbazone Derivatives.
C.C. Garcia, B.N. Brousse, M.J. Carlucci, A.G. Moglioni, M.M. Alho, G.Y. Moltrasio, N.B. D'Accorso, and E.B. Damonte.
Universidad de Buenos Aires, Buenos Aires, Argentina.

162. Construction of Pseudorabies Virus (PRV) Virion Harboring Fc Region of Mouse IgG on its Envelope.
Y. Takashima, Y. Matsumoto, and H. Otsuka.
The University of Tokyo, Tokyo, Japan.

163. Cell-Based Screening for Antivirals Against Negative-Strand Viruses.
J. Dyall, B. Buscher, G.W. Franklin, and P.D. Olivo.
Apath LLC, Saint Louis, Missouri, USA.

164. The Potentiation of Interferon-α and -β Antiviral Activities with Hydroxocobalamin Against Vesticular Stomatitis Virus (VZV), Herpes Simplex Virus Type 2 (HSV-2), and Human Hepatitis B Virus (HBV).
Y. Gong, A. Pastrak, I. Gadawski, D. Cheung, T. Tam, T. Cruz, and S. Sacks.
Viridae Clinical Sciences, Inc., Vancouver, B.C.; Transition Therapeutics, Inc., Toronto, Ontario; and The University of British Columbia, Vancouver, B.C., Canada.

165. Discovery of the First Inhibitors of the Cooperative Binding of Human Papillomavirus Type 11 E1 and E2 Proteins.
C. Yoakim, P.W. White, W. W. Ogilvie, N. Goudreau, G.A. McGibbon, J.A. O'Meara, J. Naud, B. Haché, L. Doyon, M.G. Cordingley, and J. Archambault.
Boehringer Ingelheim (Canada), Ltd., Laval, Quebec, Canada.

167. Identification of Hepatitis C Virus NS5B RNA-dependent RNA Polymerase Inhibitors from High Throughput Screening Assay.
M.K. Lee, H.S. Kim, J.-R. Choi, Y.Z. Kim, W.Y. Cho, Y.G. Cho, G.W. Kim, T.G. Kim, J.-T. Hwang, D.G. Cho, K.Y. Roh, H.S. Yoon, H.S. Lee, H.J. Kim, and J. Kim.
LG Life Sciences, Daejeon, Republic of Korea.

Thursday, May 1

Oral Session VII: Respiratory Viruses, Late Breaker Presentations

Chairpersons: Fred Hayden, Robert W. Sidwell

8:30am Plenary Speaker
Christopher Basler, Mt. Sinai School of Medicine, New York, N.Y., USA
"Existing Antivirals are Effective Against Influenza Viruses with Genes from the 1918 Pandemic Virus"

9:00 168. Inhibition of Human Rhinovirus (HRV) Infection by a Tetravalent Anti-ICAM-1 Fab Fusion Protein, CFHY196.
C.H. Charles, G.X. Luo, L.A. Kohlstaedt, L. Cao, J.H. Williams, G. Husted, A. Phillips, I.G. Morantte, E. Gorfain, A. Miller, O. Turan, and F. Fang.
Perlan Therapeutics, San Diego, Calif., USA.

9:15 169. Inhibition of Respiratory Syncytial Virus Fusion by the Small Molecule, VP-14637 via Specific Interactions with the F Protein.
J.L. Douglas, M.L. Panis, E. Ho, K.-Y. Lin, S.H. Krawczyk, D.M. Grant, R. Cai, S. Swaminathan, and T. Cihlar.
Gilead Sciences, Foster City, Calif., USA.

9:30 170. Efficacy and Tolerability of Long-Term Pleconaril Chemoprophylaxis for Picornavirus Illness in Adults.
F.G. Hayden, S. Liu, S.A. Villano, and M. McKinlay.
University of Virginia, Charlottesville, Va.; and ViroPharma, Inc., Exton, Pa., USA.

9:45 171. Arenavirus Inactivation with Conservation of Virion Surface Glycoproteins and Blockade in Viral Transcription.
C.C. Garcia, N.A. Candurra, and E.B. Damonte.
Universidad de Buenos Aires, Buenos Aires, Argentina.

10:00 Break

10:30 172. LB80380, a Novel Nucleoside Phosphonate, is a Very Potent Anti-HBV Agent.
J. Kim, J.-R. Choi, Y.Z. Kim, S.H. Lee, S. Lee, and I.-C. Kim.
LG Life Sciences, Daejeon, Republic of Korea.

10:45 Late Breaker Presentation

11:00 Late Breaker Presentation

11:15 Late Breaker Presentation

11:30 Late Breaker Presentation

11:45 Late Breaker Presentation

12:00 Conference Adjourns, Free Afternoon

Oral Session I: Retroviruses

1

"LOCK-IN" MODIFIED *cyclo*SAL-D4TMP AND BVDUMP TRIESTERS – EXTENTION OF A PRONUCLEOTIDE SYSTEM

C. Meier[#], M. Ruppel[#], D. Vukodinovic[#], A. Meerbach[‡], P. Wutzler[‡], E. De Clercq[¶] and J. Balzarini[¶]

#) Institute of Organic Chemistry, University of Hamburg, Hamburg, Germany; ‡) Institute for Antiviral Chemotherapy, University of Jena, Jena, Germany; ¶) Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium

In previous work we developed successfully the so-called *cyclo*Sal-pronucleotide system. A salicyl alcohol is used as masking unit of the phosphate group connected through a phenyl and a benzyl phosphate ester bond. The mask is cleaved by a chemically induced cascade reaction. This concept has been applied to different antivirally active nucleoside analogs targeted against RNA and DNA viruses. The *intracellular* delivery of nucleotides has also been proven by using radiolabeled *cyclo*Sal-derivatives. Moreover, *cyclo*Sal-d4TMP triesters were found to be entirely independent of the presence of cellular thymidine-kinase (TK) (retention of antiviral activity in TK-deficient CEM cells) and *cyclo*Sal-BVDUMP triesters were found to be antivirally active against EBV under conditions where BVDU was devoid of any activity. A conceptional extension of the *cyclo*Sal-technology may be an intracellular trapping of the lipophilic triester prior to hydrolysis. This would allow a so-called "lock-in" of the nucleotide releasing triester. Therefore, *cyclo*Sal-masking units have been developed in which an attached lipophilic group can be cleaved enzymatically inside the cell liberating a highly polar group, e.g. a carboxylate. This new concept has been applied to the nucleosides d4T and BVDU. The synthesis, physicochemical properties and antiviral activity data will be presented and discussed in relation to the results of the stability studies in chemical media and cell extracts. In some cases profound anti-HIV activity has been found.

2

Anti-HIV Activity Profile of AMD070, an Orally Bioavailable CXCR4 Antagonist

D. Schols[1], S. Claes[1], S. Hatse[1], K. Princen[1], K. Vermeire[1], E. De Clercq[1], R. Skerlj[2], G. Bridger[2] and G. Calandra[2]
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The antiviral efficacy of the CXCR4 antagonist AMD3100 was recently shown in a phase IIa clinical study, where the compound was given as a 10-day continuous infusion. This study demonstrated that CXCR4 is a viable target for antiretroviral therapy. Here, we evaluated the in vitro anti-HIV activity profile of AMD070, a novel orally bioavailable CXCR4 antagonist. AMD070 was examined for its activity against a wide variety of HIV-1 and HIV-2 laboratory strains (R5, X4, R5/X4), primary clinical isolates and drug-resistant viruses in different T-cell lines, CXCR4-transfected cell lines and PBMCs. Chemokine binding, chemokine-induced Ca^{2+} signaling and chemotaxis assays were performed to demonstrate the specific interaction of AMD070 with CXCR4. AMD070 was found to act as a potent CXCR4 antagonist which strongly inhibited virus infectivity at a 50% effective concentration (EC_{50}) of 1-10 nM. The compound inhibited X4 HIV replication in 5 different $CD4^+$ T cell lines, CXCR4-transfected cell lines and PBMCs. AMD070 had no activity against R5 HIV-1 variants. However, R5X4 and R3R5X4 HIV strains, which were able to use CCR3 and/or CCR5, in addition to CXCR4, for entering transfected cells, were prevented from infecting PBMCs in the presence of AMD070. AMD070 was additive or synergistic when evaluated in combination with other known HIV inhibitors such as fusion inhibitors (T-20), RT inhibitors (zidovudine, tenofovir) and protease inhibitors (amprenavir). The compound was equally active against NRTI-, NNRTI-, and PI-resistant viruses that use CXCR4 for entry. Its anti-HIV potency correlated closely with its potency in inhibiting SDF-1 binding, Ca^{2+} signaling and chemotaxis. AMD070 does not interact with any other chemokine receptor (other than CXCR4) examined to date. AMD070 holds great promise as a candidate anti-HIV drug and a clinical phase I trial with the compound is planned for the first half of 2003.

3

Persistence of Antiretroviral Activity in Chronically Infected Cells Following a Short-Term Exposure to HIV-1 Protease Inhibitors (PIs).

A. S. Mulato, G.-X. He, E. Eisenberg and T. Cihlar*. Gilead Sciences, Foster City, CA 94404, USA.

Background: Information about the correlation between intracellular drug concentration and persistence of antiretroviral activity as well as the underlying mechanisms may be helpful for understanding the dosing limitations for specific classes of HIV inhibitors. We have utilized chronically infected cells to compare these characteristics among different PIs.

Methods: PM-1 cells chronically infected with HIV-1(MN) were treated with varying concentrations of PIs either continually or for 1 hour with a subsequent removal of the drug. In both cases, infectious virus was quantified 24 hours after the initiation of treatment and the concentration reducing virus production by 50% (EC_{50}24h and EC_{50}1h) was determined for each PI. Relative persistence of the antiviral activity (P_{REL}) was calculated as the ratio of EC_{50}24h/EC_{50}1h. The concentration of cell-associated drug in extracts from PI-treated cells was measured using a calibrated HIV protease inhibition assay. The interaction of PIs with membrane phospholipids was assessed by immobilized artificial membrane chromatography (IAMC).

Results: Only nelfinavir (NFV), saquinavir (SQV), and lopinavir (LPV) exhibited a measurable persistence of activity with EC_{50}1h values of 3.2, 3.6, and 50 uM, respectively, and P_{REL} values of 0.014, 0.008, and 0.001, respectively. Despite their significant activity (EC_{50}24h = 50-350 nM), amprenavir (APV), ritonavir (RTV), indinavir (IDV) and tipranavir (TPV) did not show any measurable persistence of the activity (EC_{50}1h > 50 uM). The amount of cell-associated drug following 1-hour exposure was NFV > SQV > APV = IDV > RTV > LPV > TPV. Consistent with the persistence of antiviral activity, only NFV and SQV, but not the other PIs were detectable in PM-1 cells 18 hours after drug removal. NFV and SQV also exhibited the highest affinity to phospholipids as determined by IAMC.

Conclusion: Following a short-term exposure, NFV and SQV showed the highest intracellular loading and the most persistent antiviral effect in chronically infected cells. Their interaction with cell membrane phospholipids may contribute to generating a larger reservoir of cell-associated drug relative to other PIs.

4

Structure-function relationship of new aminoglycoside-arginine conjugates (AAC) as inhibitors of HIV-1 entry step.

A. Lapidot[‡]*, V.Vijayabaskar [‡], H.H.Lara[§], A.Kalinkovich[§], and G.Borkow[§]. [‡]Dept. of Organic Chemistry, Weizmann Inst. of Science, [§]Kaplan Med. Center, 76100 Rehovot, Israel.

Advances in understanding of HIV-1 cell entry and appreciation of the challenge of HIV-1 infection as the limitation of current therapy are recognized and defined. Novel modes of action offering new classes of drugs targeting unique elements of the virus life cycle are most important, including HIV-1 Tat-CXCR4 interaction. Although the HIV entry process was one of the earliest mechanisms examined as a target for therapeutic intervention progress was difficult and success elusive. We have recently synthesized aminoglycoside-arginine conjugates (AAC) as potential anti HIV-1 agents; they were designed to bind HIV-1 TAR and to inhibit transactivation by Tat. AAC exert a number of activities related to Tat antagonism such as increased viral production, inhibition of CXCR4 expression, etc[1-3]. The structure-function relationship for AAC is important for drug development. Thus several neomycin class antibiotics: neomycin B, paromomycin and neamine conjugated to several arginines [1-6] were synthesized[4]. Their binding to HIV-1 RNAs[4] and their anti-viral activity in HIV-1 susceptible human cell lines was investigated. Their uptake by several human cell lines was detected by using FITC-AAC. The capacity of the new AAC to block 12G5 monoclonal Ab to CXCR4 on PBMC was found for all the AAC with decreasing ability of the mono- and di-arginine derivatives of the aminoglycoside in comparison to the multi-arginine conjugates, whereas the free antibiotics have not revealed any activity. We also found that the natural ligand to CXCR4, SDF-1□ efficiently competes with hexa-arginine neomycin (NeoR6) binding to CXCR4, but much less with the other AAC. Similarly, HIV-1 protein gp-120, capable of binding to CXCR4 via CD4, reduces the AAC capacity to penetrate cells, mainly of NeoR6 and R3G. The appearance of mutations in HIV-1 gp-120 gene, in NeoR6 and R3G resistant HIV-1 isolates, supports that AAC inhibit HIV-1 via interference of HIV-1 binding to the cells. Our results point that NeoR6 is the most active AAC indicating that the number of arginine group attached to the aminoglycoside is important as well as the core itself. Neomycin, Paromomycin and neamine arginine conjugates play an important role not only as HIV-1 RNA binders but also as inhibitors of viral entry into host cells.

Ref: 1. Litovchick et al (1999) FEBS Lett. 445, 73-79; 2. Litovchick et al (2000) Biochemistry 39, 2838-2852; 3. Litovchick et al (2001) Biochemistry 40, 15612-15623; 4. Vijayabaskar et al (2002, submitted for publication).

5

Cellular factors involved in the acquisition of drug-resistance to antiretroviral drugs.

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Cellular factors have been proposed to contribute to the waning efficiency of chemotherapy. Specifically previous study have demonstrated that prolonged treatment with AZT, ddC or PMEA may favour the selection of cell resistant to antiviral activity of the respective drugs.To gain new insights into this phenomenon, cellular lines resistant to different nucleoside analogues (NA) have been developed. Specifically cells resistant to antiviral activity of 3TC (CEM_{3TC}) and d4T (CEM_{d4T}) were obtained. Kinetics experiments performed to determine the intracellular accumulation of NA indicate that, at each point tested (1, 2, 4 hours), in resistant line there is a reduction ($p<0.005$) of the drug concentration inside the cells. In order to identify the mechanism/s underlying this phenomenon enzymatic assays were performed to evaluate the activity of deoxycytidine kinase (dCK) and thymidine kinase (TK) in CEM_{3TC} and in CEM_{d4T} respectively. The results indicate that the CEM_{d4T} exhibits a 3-fold decrease in TK1 activity as compared to the parental line. On the contrary, in CEM_{3TC} cells, the enzymatic activity of dCK is similar to control cells suggesting that the changes leading to drug resistance are different. Further studies indicated that CEM_{3TC} retained much less 3TC. In order to evaluate whether an ABC protein can be involved in the efflux of 3TC, the expression of Pgp, MRP1 and MRP4 in CEM and CEM_{3TC} was evaluate. However, despite a small overexpression of MRP4, additional studies with cells specifically engineered to overexpress MRP4 demonstrated it did not impact either 3TC accumulation or efflux. Finally, a modest increase in expression of the MRP5 homologue, ABCC11 was observed in the CEM_{3TC} cells. Thus, decreased 3TC accumulation in the CEM_{3TC} appears independent of MRP4, but may be attributable to other transporters such as the recently described homologue of MRP5, ABCC11.

6

Resistance Analyses of Virologic Failure through 48 Weeks among Treatment-Naïve Patients Taking Tenofovir DF (TDF) or Stavudine (d4T) with Lamivudine (3TC) and Efavirenz (EFV)

DJ McColl, NA Margot, S Tran, DF Coakley, AK Cheng and MD Miller; Gilead Sciences, Inc., Foster City, California, 94404 USA

The K65R mutation in HIV RT has been selected *in vitro* by tenofovir and developed at low incidence (3%) in treatment-experienced patients undergoing TDF therapy. Study 903 is a 3 year, randomized, double-blind, active-controlled study of TDF therapy in 600 treatment-naïve patients. Patients received either TDF (n=299) or d4T (n=301) with 3TC and EFV. HIV from patients with virologic failure by week 48 (>400 copies/mL) was analyzed genotypically and phenotypically (ViroLogic). In this analysis, 54 patients were classified as virologic failures (9% of all patients; 29 TDF, 25 d4T) with no significant difference between treatment arms (p=0.57). Resistance to EFV or 3TC was observed most frequently (4.7% and 3.3%, resp.) without significant difference between arms ($p\geq0.37$). The K65R mutation developed in 7 TDF-treated patients (2.3%) versus 2 patients (0.7%) in the d4T arm (p=0.10). Development of EFV or 3TC resistance preceded or was coincident with development of K65R. Among TDF-treated patients with K65R, the mean HIV RNA at failure was 0.9 $\log_{10}$ below baseline, consistent with the decreased *in vitro* replication capacity of the K65R mutant virus. From *in vitro* phenotypic analyses, there were low-level changes in susceptibility to tenofovir (mean 1.4-fold), increased susceptibility for AZT (mean 0.5 fold) and low-level changes for other NRTIs; the patients without K65R showed no changes in tenofovir susceptibility. All 7 TDF-treated patients with K65R began a new regimen with a PI and other NRTIs and 5/7 achieved <50 copies/mL of HIV RNA (median follow-up 50 weeks); 2 of these fully suppressed patients had maintained TDF. In conclusion, among treatment-naïve patients virologic failure to the regimen of TDF+3TC+EFV occurred in <10% of patients by week 48 and was associated most frequently with EFV- and 3TC-associated mutations. The K65R mutation occurred in 2.3% of TDF-treated patients and there was no evidence for other TDF resistance pathways. Good virologic outcome was achieved in patients who failed with K65R and other regimen-associated resistance mutations with 2nd line therapy that included a protease inhibitor.

7

A Phase IIB Prospective, Randomized, Controlled Study Evaluating AMPLIGEN During Structured Treatment Interruption (STI) of HAART in HIV Infection. W. Mitchell[a], G. Blick[b], , D. Strayer[c], W. Carter[c] and AMP 720 Investigators*. Vanderbilt University[a], Nashville, TN; Circle Medical[b], Norwalk, CT; Hemispherx Biopharma[c], Philadelphia, PA (USA).

Background: Prolonged use of highly active antiretroviral therapy (HAART) has been associated with long term, potentially fatal, toxicities. Structured treatment interruption (STI) of HAART in chronically HIV-infected individuals to augment protective Th1 immune responses in order to achieve prolonged control of HIV-1 viremia has proven disappointing. AMPLIGEN, a double stranded RNA molecule [poly(I):poly(C_{12}U)], is a biological response modifier with anti-HIV activity and strong Th1 immunomodulatory properties that has been shown to augment cellular immune responses in HIV disease and may delay rebound of HIV during STI of HAART. **Methods:** In this Phase IIB prospective, randomized controlled study (AMP 720), individuals with HIV RNA (PCR) <50c/ml and CD4 >400 cells/mm^3 are randomized 1:1 to undergo up to three STIs with AMPLIGEN (400 mg IV twice weekly) or without (CONTROL). STI is discontinued when PCR rebounds $\geq$ 5000 c/ml for three consecutive weekly determinations or $\geq$ 50,000 c/ml once. **Results:** After a median duration of 44 + weeks, CONTROL patients (n=9) have undergone STI#1 for a median duration of 7 weeks (mean 13 weeks), while STI#1 in AMPLIGEN patients (n=7) has been significantly ($p<0.05$) prolonged to a median 15+ weeks (mean 25+ weeks). CD4 levels were statistically unchanged in either the CONTROL or AMPLIGEN arms. CD8 cells were statistically unchanged in the CONTROL arm while the mean value increased from 938 to 1130 cells/mm^3 in the AMPLIGEN arm ($p<0.01$). Adverse events with AMPLIGEN have been mild and self-limiting, and no adverse effects on lactic acid, insulin resistance, or hyperlipidemia have been observed. **Conclusion:** Initial results demonstrate that AMPLIGEN is generally well tolerated, may increase significantly the level of CD8 cells, and may prolong significantly the duration of controlled HIV-1 viremia during STI of HAART.

Oral Session II: Hepadnaviruses, Retroviruses

8

A Novel Phenotypic Assay For Monitoring HBV Drug Resistance During Antiviral Treatment

D Durantel, S Durantel, C Pichoud, B Werle, MN Brunelle, C Trépo, F Zoulim.
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Objective: HBV drug resistance is a major problem during antiviral therapy. Therefore our aim was to design new and fast cloning strategies to study the evolution of the replication capacity and the drug susceptibility of HBV genomes isolated from patients undergoing lamivudine and/or adefovir treatment.

Methods: Viral DNA was extracted from sera, PCR amplified, then cloned in more-than-full-length configuration (1.1 or 1.3 genome unit) into two different types of vectors that enable, after Huh7 cell transfection, the initiation of the intracellular HBV cycle. Northern and Southern blots analysis were performed to characterise the expression of viral RNAs and DNA replicative intermediates for HBV clinical isolates and compare their replication capacity with that of wild type strains.

Results: A detailed study regarding the biological properties and drug susceptibility, i.e. variation of IC50s and 90s, of naturally occuring HBV mutants has been performed. Throughout the course of therapy, HBV genomes from patients were cloned and evaluated for their replication competence *in vitro*. For each replication-competent clone obtained the level of replication and sensitivity to antivirals were related to the genotypes and the pattern of mutations. Multiclonal analysis enabled to evaluate the sensitivity of viral quasi-species to different drugs (3TC, PMEA, FTC, L-FMAU, PMPA) by determining IC50s/IC90s.

Conclusions: Our phenotypic assay enables the characterisation of the sensitivity to different antivirals of a viral population in less than 4 weeks. This assay will enable a better monitoring and adaptation of antiviral therapy, and will provide a new tool for the study of the molecular biology of HBV clinical isolates.

9

Synthetic and natural glycolipids induce 2-5-OA synthetase transcripts and have direct antiviral activity against hepatitis B virus (HBV)

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Alpha gal ceramide has been reported to reduce the amount of hepatitis B virus (HBV) DNA detected in the serum and liver tissue of transgenic mice, following intravenous delivery. The mechanism whereby this antiviral effect was achieved is not clear, but is thought to involve activation of an arm of the cellular immune system (NKT cells). In this report, alpha gal ceramide and smaller alkyl alpha gal molecules, were shown to be potent inducers of transcripts associated with the 2-5-OA synthetase pathway and production of interferon beta in HBV producing cells, in tissue culture. This activity parallels a direct and selective antiviral activity against HBV. Briefly, incubation of the HBV producing Hep G2 2.2.15, with as little as 1 nM alpha gal ceramide resulted in 20 fold stimulation of the p40 transcript and a greater then 90% reduction in the amount of viral DNA detected in the medium. Since, under the same conditions used for the antiviral experiments, this is a concentration that had no detectable impact upon cell viability, the activity is selectively antiviral. Curiously, the antiviral activity was transient and parabolic, with activity declining with longer times of incubation and higher concentrations. sAg was not detectably affected, under conditions where reductions in viral DNA secretion were great. Antiviral activity was found to be sensitive to formulation, with water-soluble forms far more effective than organic. The implications regarding mechanism of action, the role of glycolipids as mediators of cellular antiviral defense systems and therapeutic implications are discussed.

10

Characterisation and Clinical Significance of Lamivudine Resistant Hepatitis B Virus (HBV) Mutations in Human Immunodeficiency Virus (HIV) and HBV Co-Infected Individuals

Bartholomeusz A [1], Locarnini S [1], Ayres A , Cooley L [1,2], Lewin S [2], Crowe S [4], Mijch A [3], Roberts S [3], Sasadeusz J [2,3]

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Aims: The aims of this study were to investigate HBV/HIV co-infected individuals receiving LMV as a component of HAART therapy to; 1) determine the prevalence of genotypic resistance, 2) delineate those risk factors associated with resistance, and 3) characterise the pattern of LMV resistant HBV mutations.

Patients and Methods: Thirty three HBV/HIV co-infected individuals who fulfilled the study criteria and were treated with LMV for a minimum of six months were included in the study. HBV DNA PCR, sequencing of the HBV polymerase gene, basal core promoter and precore regions, and HBV viral load quantitation was performed.

Results: Three groups of patients were identified on the basis of HBV viral load and sequence analysis: Group 1 consisted of 11 patients (33%) who were viraemic in the absence of polymerase gene mutations, Group 2 consisted of 13 patients (39%) who demonstrated mutations in the HBV polymerase gene associated with LMV resistance and Group 3 consisted of 9 patients (27%) who were not viraemic. A high HBV DNA viral load and high CD4 count were the major factors associated with the emergence of LMV resistance. The level of serum ALT was statistically significantly higher in the group 2 patients (viraemic with LMV resistant HBV) compared to groups 1 and 3. A number of unique mutations were detected, including the triple combination rtV/G/A173L/V + rtL180M + rtM204V which was seen in three individuals and which has the *in vitro* characteristics of a vaccine escape mutant HBV.

Conclusion: HBV LMV resistance was found in 39% of HIV-HBV co-infected individuals and was associated with raised serum ALT. These patients exhibited high HBV viral loads and CD4 counts. Three patients were infected with a LMV-resistant HBV strain which could be potentially transmissible to vaccinated individuals.

11

Identification of Pre-treatment HBV DNA Markers that are Predictive of Response to Lamivudine Therapy in Patients Infected with HBV Precore Variants. B. Korba[1], A. Smedile[2], M. Rizzetto[2], M. Lagget[2], J. Gerin[1], and A. Ciancio[2] 1-Georgetown Univ., Rockville, MD USA 2-Dept. of Gastroenterology, Ospedale S. Giovanni Battista di Torino, Italy

Hepatitis B e antigen-negative (HBeAg) chronic hepatitis B represents a difficult to treat clinical liver disease that generally responds poorly to licenced therapies. Published reports have shown that reversion to wild-type at the precore/core/promoter sequences responsible for the HBe-negative phenotype (A1762T, G1764A, G1896A) occurs in a significant fraction (25-30%) of treated individuals. We examined DNA sequences of the HBV polymerase and the HBV precore/core coding regions in 26 patients with HBe-negative infections (genotype D) treated for 21-48 months with lamivudine to determine relationships between specific HBV DNA sequence patterns and long-term treatment response, and further define the effect of therapy on HBV precore mutations. Seven patients (27%) were responders (normal ALT, HBV DNA^-), 12 (46%) breakthroughs (following initial virologic/liver disease response), and 7 (27%) non-responders (sustained elevated ALT, HBV DNA^+). Two separate DNA polymorphisms in the A and E domains of the HBV polymerase present in the pretreatment serum samples were observed in 17/19 patients with extended treatment failures (serologic virus breakthrough or non-response) and were not observed in any of the 7 responders. Under therapy, reversion of A1762T/G1764A was observed in 4 of 13 patients, and reversion of G1896A occurred in 3 of 12 patients for whom DNA sequences could be obtained during therapy. None of the patients became HBeAg-positive. Mutations in the YMDD polymerase motif (confirmed by INNO-LiPA HBV DR™ analysis) were found in 7/12 of the relapsing patients at the time of virus breakthrough. A review of the literature demonstrates that, on average, over 25% of virologic breakthroughs under lamivudine therapy are not associated with mutations in the YMDD motif. Our data indicate that specific HBV DNA polymerase sequence patterns present prior to the initiation of therapy may potentially predict long-term drug response, permitting therapy to be targeted to those most likely to have maximal benefit.

12

Resistance Surveillance in HBeAg- Chronic Hepatitis B Patients (CHB) Patients Treated with Adefovir Dipivoxil (ADV) for Two Years. C.S. Gibbs[1], S. Xiong[1], H. Yang[1], C.E. Westland[1], W.E. Delaney IV[1], D. Colledge[2], A. Bartholomeusz[2], V. Thibault[3], Y. Benhamou[3], P. Angus[4], M. Wulfsohn[1], J. Fry[1], C. L. Brosgart[1] and S. Locarnini[2]. [1]Gilead Sciences, Foster City, CA USA, [2]VIDRL, N. Melbourne, Australia, [3]Hospital Pitie-Salpetriere, Paris, France, [4]Austin and Repatriation Medical Center, Heidelberg, Australia.

Introduction: HBV resistance to lamivudine occurs in 14-32% and 38-50% of CHB patients after 1 and 2 years. No resistance to ADV has been previously identified in patients treated for 48 weeks (n=629) and 96 weeks (n=45). **Aim**: Monitor emergence of adefovir resistance following 96 weeks of ADV in 79 HBeAg- patients. **Methods:** Sequencing of HBV polymerase was conducted in a prospective blinded fashion for all patients with detectable HBV DNA (>1000 copies/ml, Roche Amplicor™ PCR) at baseline and week 96. *In vitro* drug susceptibility was determined using HepG2 cells transfected with patient HBV clones or Huh7 cells transfected with HBV lab strains with engineered mutations. **Results:** A novel conserved site substitution in subdomain D of HBV polymerase (rtN236T) was identified to emerge at week 96 in two patients during blinded resistance surveillance and was independently observed to emerge, by a separate lab, in one of the same patients who displayed increasing serum HBV DNA. Patient-derived HBV and lab strains carrying rtN236T showed 6- to 14-fold reduced susceptibility to adefovir *in vitro* but appeared susceptible to lamivudine. The two patients developing rtN236T both had sub-optimal HBV DNA suppression at week 48 (~$1.7\log_{10}$) and a slow increase to within $1\log_{10}$ of baseline by week 96; 1 patient switched to lamivudine and HBV DNA suppression was observed. **Conclusions:** A novel HBV polymerase mutation rtN236T conferring reduced susceptibility to adefovir was identified in 2 HBeAg- patients taking ADV for 2 years. HBV with this mutation remained susceptible to lamivudine *in vitro* and *in vivo*. ADV has a favorable resistance profile with infrequent and delayed emergence of HBV with reduced susceptibility (0/629 patients at 48 weeks; 2/124[1.6%] patients at 96 weeks).

13

Quantitative Analyses Of Hepatic HBV cccDNA During the Natural History Of Chronic Hepatitis B and Adefovir Dipivoxil Therapy: Results Of An International Multicenter Study.

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Hepatitis B virus (HBV) covalently closed circular (ccc) DNA is a critical viral replicative intermediate responsible for viral persistence during chronic infection and following antiviral therapy. Historically, cccDNA has been difficult to assess in patients due to the low copy number in infected cells and limited access to liver biopsies. We have developed a highly-selective real-time PCR assay that allows the specific quantification of cccDNA in liver biopsies; in contrast, no cccDNA could be detected in the serum of viremic patients. This assay was used to determine levels of cccDNA patients during different phases of the natural history of chronic infection (63 patients with HBeAg+ chronic HBV, 18 with HBeAg- chronic HBV, 10 inactive carriers, and 7 with HBsAg clearance). Median levels of cccDNA in HBeAg+ patients were significantly greater (90-fold) than in HBeAg- patients or in inactive carriers ($p<0.0001$). Patients with evidence of HBsAg clearance had very low median levels of cccDNA (0.003 copies/cell). We also investigated the effect of 48 weeks of adefovir dipivoxil (ADV) therapy on cccDNA in 32 patients (22 treated, 10 placebo). ADV therapy resulted in a mean 86% decrease in cccDNA ($p=0.002$ vs. placebo). In contrast, no significant changes in cccDNA were observed in the placebo group during the 48 week trial. In conclusion, a sensitive assay for the quantification of cccDNA in biopsy samples has been established and used to generate new information on the levels of cccDNA during the natural history of chronic hepatitis B. Adefovir dipivoxil therapy results in a significant decrease in hepatic cccDNA after 48 weeks of treatment. Further analyses are warranted to determine if cccDNA can serve as an independent predictor of therapeutic efficacy and outcome.

14

RNA interference of HIV coreceptors

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Duplexes of 21bp-RNAs, known as short-interfering RNAs (siRNA), have been shown to inhibit gene expression by a sequence-specific RNA degradation mechanism termed RNA interference (RNAi). The objective of our study was to evaluate the effect of chemokine receptor gene suppression by RNAi on the entry and replication of HIV-1. siRNA that target CXCR4 and CCR5 could effectively impede cell surface protein expression and their consequent function as HIV coreceptors. The inhibitory effect of RNAi directed to CXCR4 was detected 48h after transfection of CXCR4+ U87-CD4+ cells. Expression of CXCR4 or CCR5 was blocked in up to 80% of positive cells by the corresponding siRNA. However, siRNA directed to CXCR4 or CCR5 did not have an effect on CD4 or green fluorescence expression (GFP). siRNA directed to CXCR4 did not suppress CCR5 expression or *vice versa*. Suppression of HIV-1 coreceptor expression effectively blocked HIV entry as evaluated by quantitative PCR analysis or the acute infection of CXCR4+ or CCR5+ U87-CD4+ cells by X4 (NL4-3) or R5 (BaL) HIV-1 strains. Our results demonstrate that RNAi may be used to block HIV entry and replication through blockade of cellular gene expression. Gene silencing by siRNA may become a valid alternative for HIV intervention.

15

Specific silencing of HIV-1 *env* gene using small interfering RNAs in mammalian cells

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RNA interference (RNAi) is triggered by the presence of a double-stranded RNA (dsRNA) in the cell and results in the silencing of homologous gene expression by the specific degradation of an mRNA containing the same sequence. dsRNA-mediated RNAi can be used in a wide variety of eukaryotes to induce sequence-specific inhibition of gene expression. Synthetic 21-23 nt small interfering RNAs (siRNAs) with 2-nt 3' overhangs were recently found to mediate efficient sequence-specific mRNA degradation in mammalian cells. Here, we show that synthetic siRNAs targeted against the viral structural Env proteins encoded by HIV-1 can specifically suppress the expression of HIV-1 genes. The siRNA-mediated RNAi also had advantages over antisense RNA, in terms of both the ease of design of effective antiviral agents and their potency. Especially, our best *env*-specific siRNAs, E7145 targeted to the central region of the V3 loop and E7490 targeted to the CD4 binding site of conserved regions on gp120, were significantly effective against HIV-1 replication in a human $CD4^+$ T-cell line over a 7 day period. Therefore, the use of synthetic siRNAs provides a simple, rapid, and cost-effective tool for new anti-HIV-1 gene therapeutics.

Ref. W-S. Park et al. 2002, Nucleic Acids Res. 30, 4830-4835

Poster Session I: Retroviruses, Hepadnaviruses

16

Synthesis and Anti-HIV Activities of *L*-β-3'-Fluoro-2',3'-dideoxy-2',3'-didehydro-4'-thio-nucleosides

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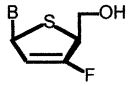
Recently, a number of nucleosides with the unnatural L-configuration have emerged as potent antiviral agents against HIV and HBV. 4'-Thionucleosides show interesting biological activity. Thus, it was important to synthesize L-4'-thio nucleosides as potential antiviral agents. In connection with L-4'-thio-nucleosides, we have recently reported the synthesis and antiviral activity of L-2'F-4'S-d4N. Herein, we report the synthesis and anti-HIV activity of L-3'F-4'S-d4N. To synthesize the target compounds, L-3'F-4'S-d4N, we started from 2-deoxy-D-ribose, which was converted to 2-deoxy-D-ribose to methyl 2-deoxy-5-*O*-toly-D-furan-3-ulose in 3 steps in 70% overall yield by the standard method. Treatment of the resulting compound with DAST afforded difluorinated intermediate in 79% yield. After ring opening with benzyl mercaptan and ring closing, followed by acetoxylation at C-1 to give 2-deoxy-3-deoxy-3,3-difluoro-5-*O*-tolyl-1-*O*-acetyl-4-thio-L-furanose. A series of pyrimidine and purine nucleosides were prepared through the coupling of 2-deoxy-3-deoxy-3,3-difluoro-5-*O*-tolyl-1-*O*-acetyl-4-thio-L-furanose and various silyl-protected bases in the presence of TMSOTf. The anomeric mixtures were produced after condensation. After separation, the β-isomers were further deprotected and eliminated to yield the target nucleosides. The cytosine derivative exhibited potent anti-HIV (EC_{50} 0.13 μM) activities (Supported in part by NIH AI32351, AI25899 and Veterans Affairs).



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Structure-Activity Relationships of D- and L-3'-Fluoro-2',3'-Unsaturated Nucleosides as Anti-HIV Agents.

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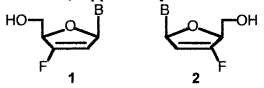
Nucleoside analogs have provided a number of antiviral agents during the past two decades. Although they are clinically effective, certain nucleosides suffer drawbacks such as toxicity and viral resistance. Among nucleoside analogs, fluorine substitution on the carbohydrate has proved effective in producing effective antiviral agents. A number of 2',3'-unsaturated analogs, such as stavudine and abacavir, are known to be effective and useful antiviral agents. We combined these two features (unsaturation and introduction of a fluorine atom) in a series of D- and L-3'-fluoro-2',3'-unsaturated nucleosides (**1** & **2**). The target compounds were prepared stereospecifically starting from chiral carbohydrate templates. The D-isomers **1** were synthesized from 2,3-*O*-isopropylidene-D-glyceraldehyde in 9 steps, and the L-isomers **2** were prepared from L-xylose in 13 steps. The synthesized compounds were evaluated for anti-HIV activity, and selected active compounds were also evaluated against lamivudine-resistant strains of HIV. Comprehensive structure-activity relationships will be presented. Molecular modeling studies of the complex of D- and L-3'-Fd4CTP with wild-type and M184V-mutated RT were performed. The calculated energies of the complexes were correlated with the observed antiviral activities, and partial resistance of the L-analog was explained in terms of steric hindrance with Val184. The model also suggests interesting interactions involving the 2',3' double bond and the 3'-fluorine atom. (Supported by NIH AI32551 and AI25899)



18

Anti-HIV Activity and Molecular Mechanism of Drug Resistance of D- and L- 2'-F-4'-S-d4N

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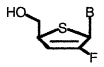
Both D- and L-2'-fluoro-4'-thio-2',3'-unsaturated nucleosides (**1** & **2**) were synthesized and their anti-HIV activity against the drug sensitive and lamivudine-resistant mutant (M184V) virus was evaluated. Among the synthesized nucleosides, cytidine, 5-fluorocytidine, adenosine and 2-fluoroadenosine analogs showed moderate to potent anti-HIV activity. D-2'-F-4'-S-d4C and L-2'-F-4'-S-d4C as well as their 5-fluorocytidine analogs confer significant cross-resistance with 3TC. The isosteric substitution of 4'-oxygen with 4'-sulfur resulted in cross-resistance with the M184V mutant. Molecular modeling studies indicated that the M184V mutation in HIV-1 RT caused a serious problem in positioning the L-configured nucleoside triphosphates at the active site because the side chain of Val184 tended to occupy the space where the sugar moiety of L-2'-F-2',3'-unsaturated nucleosides projected. The resulting steric hindrance destabilized the L-2'-F-2',3'-unsaturated nucleoside triphosphate/RT complex. It was interesting that M184V RT was cross-resistant to the natural D-2'-F-4'-S-d4C, while the corresponding D-2'-FD4C (Reverset®) showed potent activity against M184V mutant. The longer van der Waals radius of 4'-sulfur and the C-S bond length placed the 4'-sulfur atom close enough to Val184, which resulted in steric hindrance, and thereby reduced relative binding affinity and antiviral potency. Detailed synthesis, biological evaluation, and molecular modeling studies will be presented (Supported by NIH grant AI32351 and the Department of Veterans Affairs).



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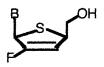

2

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Glycopeptide Antibiotic Derivatives Selectively Inhibit Human Immunodeficiency Virus Replication in Cell Culture

Jan Balzarini[1], Christophe Pannecouque[1], Sigrid Hatse[1], Dominique Schols[1], Erik De Clercq[1], Y. Pavlov[2], S.S. Printsevskaya[2] and Maria Preobrazhenskaya[2]
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The antiretroviral activity of a series of semisynthetic derivatives of natural antibacterial glycopeptide antibiotics (i.e. vancomycin, eremomycin, teicoplanin) has been investigated. The most active derivatives were represented by the antibiotic aglycone derivatives substituted with hydrophobic groups at several places on the molecules. The most potent inhibitors against human immunodeficiency virus type 1 (HIV-1) and HIV-2 had 50% effective concentration (EC_{50}) values in the lower micromolar range (1-5 μM), while not being cytostatic against T-lymphocyte cell proliferation at 250 μM. Time-of-addition experiments revealed that the compounds prevented entry of HIV into the T-lymphocytic target cells. Also, flow cytometric analysis and monoclonal antibody binding studies, and a PCR-based assay revealed that the drugs likely interrupt the viral entry process. The drugs did not interfere with monoclonal antibody binding to the CD4 receptor and CXCR4 co-receptors of HIV. In conclusion, new lipophilic derivatives of aglycons of vancomycin, eremomycin and teicoplanin represent a new class of selective inhibitors of HIV. Given the virus entry process as their plausible target of antiviral action, they should be further considered for development as potential microbicides against HIV.

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Abacavir Phosphoramidate Analogues: The Effect of Amino Acid Variations on the Biological Activity.

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[1]Rega Institute for Medical Research, Leuven, Belgium.
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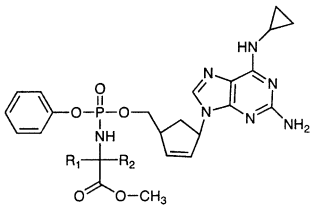

Since its discovery, the phosphoramidate technology proved to be a very efficient method to deliver biologically active nucleotide analogues into the cells, bypassing the first kinase mediated phosphorylation step. We recently report the successful application of this methodology to the GlaxoSmithKline carbocyclic nucleoside analogue abacavir. The phenyl-methoxyalanine protide derivative proved to be 100-fold more potent than the parent nucleoside against HIV. Following that work, in this presentation we will discuss the effect of the variation of the protide amino acid moiety on the biological activity. Anti-HIV as well as anti-HBV data will be presented and the different SAR profiles will be compared.

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In Vitro and *In Vivo* Activity of a Novel Small Molecule Against HIV.

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Resistance to specific HIV drugs and/or classes of drugs is rapidly emerging. Therefore, drugs with novel mechanisms of action (MOA) against HIV are urgently needed. KPC-2 is a small molecular weight compound with a novel MOA against HIV-1. The MOA of this class of compounds involves alteration in functional gp120 expression on the surface of infected cells and virus particles resulting in production of defective HIV and inhibition of infected cell-cell fusion (Krugner-Higby et al., AIDS Res. Hum Retro 11:705-712, 1995). The purpose of this study was to evaluate the *in vitro* and *in vivo* activity of KPC-2. A syncytial plaque assay was used to determine *in- vitro* activity. The efficacy/ toxicity of KPC-2 was evaluated *in vivo* using a retrovirus (Friend murine leukemia virus, FMLV) infected mouse model. The EC_{50} and TC_{50} of KPC-2 in HIV (IIIB) acutely infected CEM-SS cells were 0.03 μM and >100 μM, respectively. In HIV-1 (IIIB) chronically infected CEM-SS cells, treatment with KPC-2 (1 μM) resulted in 78% inhibition of infectious virus production. Also, KPC-2 markedly inhibited matched pairs of AZT-sensitive (EC_{50}= 0.19 μM) and AZT-resistant (EC_{50} = 0.15 μM) human clinical isolates of HIV-1 in infected CEM-SS cells. Activity against other drug resistant HIV variants is ongoing. Administration of KPC-2 (12.5, 25, and 50 mg/kg) by i.p. administration daily x 14 days to female Balb/c mice infected with FMLV resulted in a dose-dependent and significant reduction ($p<0.05$) in splenomegaly at 25 or 50 mg/kg of drug. KPC-2 was well tolerated at all doses. In summary, KPC-2 has potent *in vitro* activity against HIV-1, is active and well tolerated *in vivo* and may offer a new approach toward treatment of HIV infections in humans.

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Dinucleotide Inhibitors of HIV Integrase: Implications of Inhibitor Structure and Stereochemistry.

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The mechanism of integration of double-helical HIV DNA into host chromosomal DNA by the viral enzyme, HIV integrase, involves two major steps. The first step involves excision of the two terminal nucleotides at the 3′-end of each strand of viral DNA (3′-processing), leaving recessed ends that terminate with xxCA-OH. In the next step (strand transfer, integration), nucleophilic attack of the 3′-OH on a specific phosphodiester bond results in cleavage of host DNA and subsequent integration of the tailored HIV DNA into host DNA. Some small oligodeoxynucleotides of natural origin are capable of interfering with both steps of integration by competing with the viral DNA for binding to HIV integrase. However, these "natural" oligonucleotides are rapidly cleaved by nucleases. Increasing nuclease stability by chemical alteration of the internucleotide phosphate bond results in decreased integrase activity. We have designed and synthesized non-natural dinucleotides, with unusual internucleotide phosphate bonds, that exhibit nuclease stability and anti-HIV integrase activity. This paper will describe the progress of our work in this area including synthesis, molecular modeling studies and anti-HIV integrase activity.

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Anti-HIV Activity of Newly Cloned Secretory Leukocyte Protease Inhibitor (SLPI)

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Secretory leukocyte protease inhibitor (SLPI), a serine protease inhibitor present in saliva, has been shown to inhibit HIV infection of macrophages and primary T-cells. However, contradictory results have also been published and variable results were observed in our laboratory. All these studies have been performed using the same, commercially available, preparation of recombinant SLPI obtained from Synergen and/or R&D Systems. We examined the anti-HIV activity of a newly cloned SLPI using human macrophages and differentiated THP-1 cells. THP-1 cells were induced to differentiate by treatment with phorbol 12-myristate 13-acetate (PMA). Macrophages and THP-1/PMA cells were infected with HIV-1_{BaL}, in the absence or presence of SLPI. Cells were incubated with SLPI, for 30 min at 37°C prior to addition of the virus. SLPI was also present during the 2-h infection period. Over 90% inhibition of p24 production was observed in macrophages infected in the presence of 5, 10 or 20 µg/ml of SLPI. At 0.5 or 1 µg/ml, SLPI reduced the p24 levels by ~70 to 80%. This potent inhibition of virus production was sustained for two weeks of culture. SLPI also significantly reduced p24 production in THP-1/PMA cells. At 5 or 10 µg/ml SLPI, ~95% inhibition of p24 production at 7 day and ~90% at 11 day post-infection was observed. The p24 viral protein was undetectable after 3 and 5 days. The availability of an active SLPI will enable the investigation of the biochemical and structural basis for its anti-HIV activity.

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Synthesis of Anti-HIV CADA Compounds and Quantitative Structure-Activity Relationships for CD4 Down-Modulation

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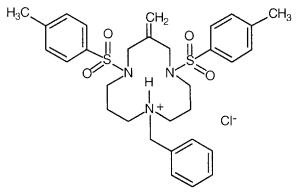
CADA (9-benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane), a novel antiviral lead compound, inhibits HIV replication at the micromolar level by decreasing the expression of the CD4 glycoprotein. CADA has no effect on any other cellular receptor, and its mechanism is completely different from that of any other anti-HIV compound described to date. We have now synthesized several new cyclic analogs of CADA and examined quantitative structure-activity relationships (QSAR) for the CD4 down-modulating activities of more than 30 CADA compounds. A new solution-phase synthesis was developed for access to unsymmetrically substituted derivatives. Some new structural units were incorporated, including fluorescent tags and carboxamide side arms. A 3-dimensional QSAR model for CD4 down-modulation by CADA compounds is also presented.



CADA

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New pyrophosphate analogues as substrates and inhibitors of HIV reverse transcriptase

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The removal of nucleotide chain-terminators by a pyrophosphate acceptor molecule has been proposed as a mechanism that causes the viral resistance to HIV nucleoside inhibitors, for example, AZT and d4T. Herein, a set of pyrophosphate analogues (pyrophosphoric, hypophosphonic, and diphosphoic acids) was synthesized and their substrate properties in the pyrophosphorolysis reaction catalyzed by HIV reverse transcriptase (RT) were investigated. Although hypophosphoric acid (O_3P-PO_3) has a 20% shorter distance between the phosphorus atoms compared with pyrophosphate (O_3P-O-PO_3), it displayed lower Km and higher Vmax than natural pyrophosphate in the pyrophosphorolysis reaction. Neither pyrophosphonic nor diphosphonic acids were substrates/inhibitors of the reaction. The dATP analogue containing a P-P fragment in the β-γ position showed substrate properties similar to dATP in the elongation reaction. The abilities of (O_3P-PO_3) and pyrophosphate to remove nucleotide derivatives (ddCMP, 3TCMP and others) from the 3'-end of primer were compared. Structure-function relations of pyrophosphate analogues towards HIV RT will be discussed. The work was supported by the Russian Foundation for basic Research, project no 02-04-48951 and ISTC, project no 1989.

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The Potential of Novel Ribonucleotide Reductase Inhibitors, Didox and Trimidox, Compared to Hydroxyurea to Enhance NRTI Antiviral Activity In Vivo. R Sumpter[1], H Elford[2], M Inayat[1], T Sugg[1], P Tsai[1], and V Gallicchio[1]. [1]U of Kentucky, Lexington, KY [2]Molecules For Health Inc., Richmond, VA, USA

Depleting deoxynucleotide pools in HIV infected cells by inhibiting ribonucleotide reductase (RR) as a strategy to impair HIV replication has gained credibility by the success of hydroxyurea (HU) to enhance the deoxynucleotide reverse transcriptase inhibitor (RTI) ddI in clinical trials. However, HU as a single agent in HIV therapy and the role of RR inhibitors (RRI) in potentiating other anti-HIV deoxynucleosides has not been demonstrated clinically. Compared to HU, the novel RRIs Didox (DX) and Trimidox (TX) have shown more potent antiviral activity when used alone in murine retroviral models and have been shown to enhance the nucleoside RTIs ddI and abacavir. Antiviral activity was more pronounced with these compounds than HU, especially in the HIV-infected SCID-Hu mouse model. This report focuses on comparing the ability of DX, TX or HU, in the MAIDS model of retrovirus disease, to potentiate the antiviral activity of an adenosine-containing deoxynucleotide RTI, tenofovir (TFV). MAIDS infected mice were treated daily (ip) starting one week after infection with monotherapy with either RRI alone or a combination of RRI and TFV with each drug at non-optimum levels. Antiviral activity was assessed by measuring spleen and lymph node size and by monitoring IgG levels. At the dosage used, the more than 4-fold increase in spleen size due to the MAIDS infection was reduced by 50% to 55% by the RRI alone. TFV monotherapy exhibited a reduction of 33%. Importantly, the combination of DX and TFV demonstrated the best antiviral effect of 65% reduction in the increase or a more than 80% reduction in the overall change in spleen size due to infection. The combination of HU or TX with TFV did not exhibit an improvement over these RRIs alone at the 4 wk interval. However, in the 8 wk treatment groups there was a modest enhancement of the antiviral effect of the RRI by the inclusion of TFV in the regimen. In conclusion, the data further support the concept of using RRIs for treatment of retrovirus infection and adds a new class, the deoxynucleotide RT antiviral agent TFV, that can be enhanced by RRIs, in particular the novel RRI Didox.

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1-Substituted 4-Methoxy-2-phenylbenzimidazoles as NNRTIs: Synthesis and Anti-HIV Evaluation

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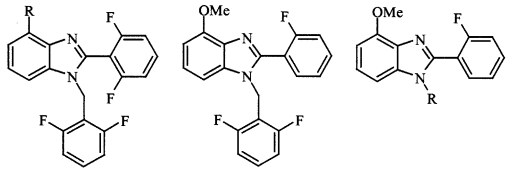
Trisubstituted benzimidazoles (**A**) have been shown to be active against HIV-1. Our previous research revealed that the 2-monofluorophenyl analogue **B** has improved anti-HIV activity and selectivity.



A (R = Me, OMe) **B** **C**

In this study we have replaced the 1-(2,6-difluorobenzyl) substituent by (substituted) benzyl, heteroarylmethyl, 2-phenylethyl, alkyl, alkenyl, etc. The compounds were evaluated for their anti-HIV activity. It could be shown that an additional fluorine in position 3 of the benzyl is beneficial for anti-HIV activity. High activity and selectivity could also be found for the 1-isopropoxycarbonyl analogue. Moreover, we found out that longer (cyclo)alkyl or alkenyl substituents also have high anti-HIV activity. As expected for NNRTIs, no activity against HIV-2 could be found.

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3,4-Dialkyoxyquinolin-2(1*H*)-ones and 4-Alkyoxy-1,6-naphthyridin-2(1*H*)-ones as HIV Nonnucleoside RT Inhibitors

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HIV-1 nonnucleoside reverse transcriptase inhibitors (NNRTIs) are part of the combination therapy currently used to treat HIV infection. The features of a new NNRTI drug for HIV treatment must include selective potent activity against wild-type virus as well as potent activity against mutant virus that result from current antiretroviral treatment regimens. Based on analogy with known HIV-1 NNRTI inhibitors and modeling studies utilizing the X-ray crystal structure of inhibitors bound in the HIV-1 RT, a series of substituted 2-quinolones was synthesized and found to be potent inhibitors of HIV-1. While selected compounds were found to inhibit wt HIV with IC_{50}s in the low nanomolar range, they were greater than 99% protein bound. Introduction of heteroatoms at the 3-position and in the 6-ring position to give 3,4-dialkyoxyquinolin-2(1*H*)-one and 4-alkyoxy-1,6-naphthyridin-2(1*H*)-one analogs was investigated as a method of reducing protein binding while maintaining antiviral potency. Unfortunately, these compounds demonstrated potency too low to be considered for development as NNRTI agents.

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Characterization of a Novel Series of Nevirapine-like Next-Generation NNRTIs with Broad Antiviral Potency Against NNRTI-Resistant HIV

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Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are potent components of combination antiretroviral therapies. However patients failing on an NNRTI containing regimen most often exhibit broad cross-resistance to all members of the class leaving them with no further NNRTI options. A pre-requisite for a next generation of NNRTI is potent antiviral activity against clinically prevalent NNRTI-resistant variants. Advanced derivatives of the NNRTI nevirapine (NVP) were used as starting point for the development of new chemical entities with broader antiviral profile. Optimization was guided by RT inhibition and cell-based viral replication assays using a panel of clinically relevant RT mutants (K103N, Y181C, G190A, etc). Key biopharmaceutical parameters (serum shift, metabolic stability, CYP450 inhibition) were also measured to assist in the selection of candidates for animal PK studies. Following extensive SAR, a series of nevirapine-like molecules with a quinoline extension at the C-8 position was identified. This series displays EC50 values < 1nM against wild-type virus and <10nM against prevalent single and double mutants, along with favorable biopharmaceutical properties. A detailed characterization of representative members of this series is provided. These compounds are valuable candidates for further development.

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Pharmacokinetics and Developability of GW4511, a novel benzophenone non-nucleoside reverse transcriptase inhibitor of HIV-1. D Reynolds, P Wheelan, C Edwards, J Tidwell, J Chan, G Freeman, K Romines, L Schaller, J. Cowan, R Ferris, D Hazen, M St. Clair, L Boone, GlaxoSmithKline, Research Triangle Park, North Carolina, USA

A cornerstone of treatment for human immunodeficiency virus (HIV) is inhibition of HIV reverse transcriptase (RT), the enzyme responsible for generating proviral DNA from viral RNA. Inhibition of the RT enzyme, accomplished by either chain-terminating nucleoside reverse transcriptase inhibitors (NRTI) or allosteric non-nucleoside reverse transcriptase inhibitors (NNRTI), blocks viral replication. Unfortunately, NNRTIs rapidly select for mutational changes in RT that confer resistance toward that agent. In order to discover and develop a new generation NNRTI, compounds must be identified that are active against strains resistant to the current NNRTI drugs, particularly the K103N and Y181C strains which are cross-resistant to the majority of NNRTIs currently utilized. GW4511 was identified from a series of benzophenone analogs as a tractable lead that demonstrated potent activity against wild-type HIV-1 and a panel of 20 NNRTI-resistant strains. Metabolic stability of GW4511 was established through in vitro incubations using rat, dog, monkey, and human liver microsomes. Its pharmacokinetic characteristics were evaluated in the rat, dog, and monkey. The calculated clearance rates of GW4511 after intravenous doses at 1 mg/kg in these species were 11, 7, and 10 mL/min/kg, respectively. The oral bioavailability of GW4511 (dosed as a solution) in rat, dog and monkey was 27%, 29% and 58%, respectively, at clinically relevant doses (1 to 10 mg/kg). Solid amorphous drug material (dosed as a micronized powder in a capsule) generated similar oral bioavailability in both rat and dog compared to GW4511 in solution. However, the oral bioavailability from a stable micronized crystalline form of GW4511 was significantly decreased in the rat. Further development of compounds from this series must provide improved oral bioavailability from crystalline drug substance.

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Prevention of HIV-1 Infection by Phthalocyanines

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The ability of selected phthalocyanines and metallophthalocyanines to block HIV infection has been evaluated in epithelial HeLa –CD4 cell line with an integrated LTR-ß-galactosidase gene. Sulfonated phthalocyanine itself (PcS), as well as its copper, nickel and vanadyl chelates, were the most effective in blocking viral infection. These compounds were also very effective in blocking the fusion activity of the viral Env proteins. All of these compounds are expected to bind axial ligands weakly or not at all. In contrast, sulfonated phthalocyanines bearing metals expected to bind axial ligands more tighly (aluminum, cobalt, chromium, iron, silicon and zinc) were less effective in blocking HIV infection and also less effective at inhibiting fusion. Active compounds also tended to block binding of gp120 to CD4. Selected cationic and carboxy phthalocyanines, as well as porphyrazines, were also evaluated. Our results indicate that at least some of the compounds render the virus noninfectious, i.e., that they are virucidal. These compounds have potential as microbicides, which might be used to provide protection against sexually transmitted HIV.

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Development of a Dual-Reporter Assay for Identifying and Characterizing HIV-1 Tat Inhibitors.

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The HIV-1 Tat protein is required for HIV-1 replication and therefore represents an excellent target for antiviral drug development. As part of our HIV-1 drug discovery efforts, we have recently developed a dual-reporter gene assay for the identification and characterization of HIV-1 Tat inhibitors. The assay was developed through the stable transfection of HeLa cells with two different protein expression cassettes. The first contains a tetracycline controllable promoter driving the expression of both HIV-1 Tat and Firefly Luciferase (FLuc) from a single bicistronic mRNA. In this format, the FLuc reporter allows for the identification of cytotoxic compounds or non-specific inhibitors of Tat expression. The second cassette contains an HIV-1 LTR-Renilla Luciferase (RLuc) reporter gene construct which requires HIV-1 Tat transactivation of the LTR for expression of RLuc. This dual-reporter gene approach allows for the identification of specific inhibitors of HIV-1 Tat function. Furthermore, the use of a tetracycline controllable promoter allows for subtle modifications in Tat expression during the characterization of inhibitor activity. A detailed description of the assay, along with assay validation data and preliminary screening results will be presented. This work is partially supported by NIH/NIAID contract N01-AI-05415.

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Suppression of HIV-1 replication by an HIV-1 dependent anti-gene expression vector with the Cre-loxP System

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We previously demonstrated the development of the HIV-1 5'LTR and the Cre/loxP system as anti-gene (U5-ribozyme and RNAi) expression vectors, with which the site-specific excision of loxP sequences can be achieved by using the Cre/loxP system as a molecular switch in an acute HIV-1 infection. However, this expression system also revealed the lower, non-specific expression of the anti-HIV-1 ribozyme in the absence pNL4-3. To circumvent this problem, we used the more efficient HIV-1 dependent Cre recombination gene expression vector, encoding the LTR-gag-p17M (intermediately region of gag p17). Furthermore, we constructed the pCre/loxP-Rz-LTR-gag-p17M as a single vector. The function of this vector is to induce the HIV-1 dependent ribozyme-mediated inhibition in a dose-responsive manner. However, the ribozyme mRNA expression was only detected in the presence of pNL4-3 in HeLa-CD4$^+$ cells. These studies have yielded a potential novel strategy for antiviral applications in HIV-1 gene therapy.

Reference: Habu Y., Miyano-Kurosaki N., Nagawa T., Matsumoto N., Takeuchi H., and Takaku H. Inhibition of HIV-1 replication by an HIV-1 dependent ribozyme expression vector with the Cre/loxP(ON/OFF) system. (2002) *Antivir. Chem. Chemother,*

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Inhibition of Human Immunodeficiency Virus Type 1 (HIV-1) by ribozyme expression baculovirus vector

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The purpose of this study is to demonstrate the capability of gene tyerapy using baculovirus vector. We constructed a recombinant baculovirus possessing Vesicular Stomatitis Virus Glycoprotein (VSV-G) gene under the polyhedrin promoter and green fluorescent protein gene under the CMV promoter. The result indicated that all of the mammalian cell line infected with VSV-G pseudotype recombinant baculovirus exhibited relatively higher expression than cell lines infected with wildtype baculovirus.

Furthermore, we constructed the LTR specific ribozyme expression baculovirus vector(AcVSV-G/LTR-Rz) for HIV-1 gene therapy of. AcVSV-G/LTR-Rz was infected to mammalian cells at the m.o.i. of 50. The result demonstrated that all of the mammalian cells infected with AcVSV-G/LTR-Rz exhibited over 90 % inhibition as compared to the MOCK infected cells. The efficiency of inhibition in all the mammalian cells showed baculovirus dose dependent. There was no cytotoxicity observed after the baculovirus infection in all of the mammalian cells. These results suggest that baculovirus has a high potential of becoming new gene therapy vector.

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Mechanism of 2',3'-Dideoxyinosine's Drug Interaction with Allopurinol, Ganciclovir and Tenofovir Disoproxil Fumarate, Ray, A.S., Mahmoudi, A., Fridland, A. Gilead Sciences, Foster City, CA, USA

The plasma concentration and area under the curve for 2',3'-dideoxyinosine (ddI) are substantially increased when it is co-administered with allopurinol, ganciclovir or tenofovir disoproxil fumarate (TDF, Viread™). A major metabolic route for ddI clearance is its breakdown by purine nucleoside phosphorylase (PNP) to 2',3'-dideoxyribose (ddR) or 2',3'-dideoxyribose-1-phosphate (ddR-1-P) and hypoxanthine. In order to test the validity of PNP inhibition as a mechanism for the clinically observed drug interaction, enzyme kinetic and cellular studies were undertaken to assess the effects of these purine analogs and their metabolites on ddI degradation by PNP. Consistent with previous reports, enzymatic inhibition assays showed that acyclic nucleotide analogs are effective inhibitors of the phosphorolysis of inosine. It was further established that the mono- and diphosphate forms of tenofovir are potent inhibitors of PNP-dependent degradation of ddI (K_i values of 38 nM and 1.3 μM, respectively). Cell culture studies in T-cells directly measuring ddI breakdown showed that allopurinol, ganciclovir and tenofovir were all able to inhibit ddR and ddR-1-P formation. Concurrently ddI concentrations increased correlating well with *in vivo* observations. Despite higher ddI levels, no significant effect on ddATP formation was observed. The lack of an increase in ddI phosphorylation in cells may, in part, explain why increases in ddI-mediated adverse effects have not been noted in clinical studies using the combination of ddI with TDF.

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In Vitro Synergy between the CD4 Down-Modulating Compound, CADA, and Multiple Anti-HIV Drugs

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The infection of target cells by HIV is mainly dependent on the presence of the CD4 surface molecule, which serves as the primary virus receptor. Therefore, drugs that target the CD4 receptor, and thus inhibit viral entry, may be promising agents for the treatment of AIDS. Here, we present the in vitro anti-HIV activity profile of CADA, a specific CD4 receptor down-modulator, alone and in combination with antiretroviral drugs. Cyclotriazadisulfonamide (CADA), inhibited HIV (R5, X4 and R5/X4) infection (EC_{50}: 0.3-3.2 μM) in T cell lines, PHA-stimulated PBMCs and monocytes/ macrophages. When the cells were pretreated with CADA for 24 hours, they became markedly protected against viral infection. Flow cytometric analysis revealed a significant decrease in the expression of the CD4 receptor. Moreover, the antiviral activity of CADA correlated with its ability to down-modulate the CD4 receptor. In addition, CADA did not alter the expression of any other cellular receptor examined so far. Further analysis of CD4 mRNA levels suggested that CADA is not involved in the regulation of CD4 expression at the transcriptional level, but most probably interferes at a (post)translational level. In addition, CADA showed synergy when evaluated in combination with other known HIV inhibitors. Synergy was seen when CADA was combined with nucleoside or nucleotide reverse transcriptase (RT) inhibitors (zidovudine, stavudine, lamivudine, zalcitabine and tenofovir) or non-nucleoside RT inhibitors (nevirapine and delavirdine). In combination with the protease inhibitors amprenavir and indinavir, CADA was slight synergistic. Furthermore, synergy was observed between CADA and other entry inhibitors such as the fusion inhibitor T-20 and the CXCR4 antagonist AMD3100. Specific CD4 down-modulating agents may be considered as promising candidate anti-HIV drug, also in conjunction with currently available antiretroviral drugs.

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AN APPROACH TO DEVELOPMENT OF ANTI-HIV AGENTS WITH DOUBLE ANTIVIRAL PROTECTION

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One from fundamental causes of reduced antiviral protective efficacy of traditional narrow-selective antivirals is viral drug resistance formation. To prevent a drug resistance we developing some approaches toward design of multi-targeted drugs, which able to stop more then one steps of infection progression: from one event to next event in chain of a viral life cycle. Earlier we reported about two novel groups of anti-HIV-1 agents acted on early stage of HIV-1 entry into cells, including adsorption (slow-downing) and fusion (blocking). The molecular basis of these agents is anionic polymeric matrixes possessed electrostatic driven inhibitory activity against HIV-1 adsorption (gp120, V3). Incorporation of hydrophobic sensitive vectors, such as bi- or tricyclic frame hydrocarbons, into the drugs macromolecular system (**MTS**) allow to effectively block the followed post-adsorption events: fusion (gp41) or/and uncoating (gag) [*Antivir. Res.1999.41(2):A45-46; 41(3):134-44; 2002.53(3):A50*]. Presently an involving of gag proteins, as possible molecular targets for MTS, is confirmed by evaluation of adamantine-containing MTS on experimental model for HIV-virus-like particles production based on gag containing recombinant variola vaccine virus *in vitro* [*Russ J HIV/AIDS 2002, 6(1):167*]. Here the novel approaches toward enhancing of the gag–targeted functions of the novel MTS-like agents directed to inhibition not only early, but also late stage of HIV-1 replication, are discussed. The main targets for additional antiviral protection are p55/p17/p24 at steps of assembly, budding and maturation. And key way of the new agents to epicenters of HIV-1 assembly on plasma membrane is associated with "raft"-tropic functions due to cholesterol derived vectors.

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Induction of cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors by breast cancer resistance protein (BCRP)

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BCRP is a novel member of ATP-binding cassette transporters, which induce multidrug resistance in cancer cells. We found that a high level of BCRP expression in $CD4^+$ T cells conferred cellular resistance to human immunodeficiency virus type 1 (HIV-1) nucleoside reverse transcriptase inhibitors. The cell line MT-4/DOX_{500} was established by long-term culture of MT-4 cells in the presence of doxorubicin (DOX) and had reduced sensitivity to not only DOX but also zidovudine (AZT). MT-4/DOX_{500} cells showed reduced intracellular accumulation and retention of DOX and increased ATP-dependent rhodamine 123 efflux. The cells were also resistant to several anticancer agents. AZT was 7.5-fold less inhibitory to HIV-1 replication in MT-4/DOX_{500} cells than in MT-4 cells. Furthermore, the anti-HIV-1 activity of lamivudine was severely impaired in MT-4/DOX_{500} cells. In contrast, the antiviral activity of non-nucleoside reverse transcriptase inhibitors and protease inhibitors was not affected in the cells. MT-4/DOX_{500} cells expressed glycosylated BCRP but not P-glycoprotein, multidrug resistance protein 1, 2, 4, or lung resistance-related protein. In addition, fumitremorgin C, a specific inhibitor of BCRP, completely abolished the resistance of MT-4/DOX_{500} cells to AZT as well as DOX. An analysis for intracellular metabolism of AZT suggests that the resistance is attributed to the increase of ATP-dependent efflux of its metabolites in MT-4/DOX_{500} cells.

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Pre-Steady state kinetic Analysis of the incorporation of FTC-5'TP and 3TC 5'-triphosphate by mutant HIV-1 RTs K65R, K65R/Q151M and Q151M. Gilbert B. Mulamba, Laurence Rimsky, Florence Myrick, Katyna Borroto-Esoda, Joy Y. Feng, Triangle Pharmaceuticals, Inc., 4611 University Drive, P.O. Box 50530, Durham, NC 27711. USA

FTC (2',3'-dideoxy-5-fluoro-3'-thiacytidine, emtricitabine) is a nucleoside analogue that is a potent and selective inhibitor of HIV and HBV replication. This antiviral agent, once converted to its 5'-triphosphate form by intracellular metabolism, inhibits HIV-1 reverse transcriptase (RT) *in vitro* and *in vivo*. The K65R mutation identified in isolates from patients treated with 2',3'– dideoxycytidine (ddC) and tenofovir (PMPA) confers 3-, 3-, 20- and 13-fold resistance to ddI, ddC, 3TC and FTC, respectively. The Q151M mutation is involved in multiple dideoxynucleoside resistance (MDNR) and confers low levels of resistance to ddI, ddC, AZT and d4T. Using MT2 cells in phenotyping assays, it was determined that the combination of K65R and Q151M mutations resulted in more than a 20-fold reduced sensitivity to 3TC and FTC. In this study, we used pre-steady state kinetics analysis to evaluate the DNA-dependent incorporation of dCTP, FTC-TP and 3TC-TP by wild-type and mutant HIV RTs containing K65R, Q151M and K65R/Q151M, respectively. Two kinetic constants k_{pol} (the maximum rate of incorporation) and K_d (reflects the binding affinity of enzyme-DNA for the analogs) were measured. For the incorporation of FTC-TP by K65R, Q151M and K65R/Q151M, kinetic data analysis shows respectively 12-, 2.4- and 64-fold decrease in k_{pol}. Similarly for 3TC-TP incorporation, k_{pol} decreased by 19-, 3- and 93-fold in K65R, Q151M and K65R/Q151M. The mutant HIV-1 K65R displayed 5-fold resistance against both FTC and 3TC. While the mutant K65R/Q151M exhibited 11-fold and 3-fold resistance to FTC-TP and 3TC-TP respectively, the single Q151M mutant displayed no resistance to either nucleoside analog. Overall, the results of these enzymatic studies are in agreement with the cell culture studies using recombinant HIV-1. These data demonstrated that the K65R and K65R/Q151M-related resistance to FTC-TP and 3TC-TP is mainly due to a significant decrease in the rate of incorporation.

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Molecular Mechanism Studies of Tenofovir against AZT & 3TC Drug Resistant Mutants.

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The molecular modeling studies of structurally diverse nucleoside HIV-1 reverse transcriptase (RT) inhibitors (AZT, 3TC and tenofovir) complexed with the wild type (WT) and mutant (M184V and D67N-K70R-T215Y) RT were conducted in order to understand the molecular mechanism and the potent antiviral activity of tenofovir against AZT- (D67N-K70R-T215Y) and 3TC (M184V) resistant mutants. The tenofovirTP maintained the high relative binding energy to both WT and M184V RT, showing its effectiveness against the 3TC-resistant mutant. The acyclic sugar moiety was found far away from the bulky side chain of Val184 to avoid the unfavorable steric hindrance. However, in the case of 3TCTP-M184V RT complex, the unnatural L-sugar conformation of 3TCTP resulted in steric hindrance with Val184, which is reflected on decrease in binding energy of 3TCTP and a wide range of conformational changes at the enzyme as well as the DNA duplex. In the AZT-resistant RT (D67N-K70R-T215Y)-AZTTP complex, the mutation (D67N and K70R) pushes the 3'-azido group of AZTTP toward the Asp185, which might cause an unfavorable steric hindrance when the AZTMP moves along the primer chain. Therefore, the AZT-resistant mutant RT effectively holds the AZTMP at the nucleoside binding site allowing better conformation for excision reaction by the pyrophosphate donor. In the AZT-resistant RT (D67N-K70R-T215Y)-tenofovirDP complex, however, the acyclic nature of the sugar moiety of the tenofovir having minimal size and torsional flexibility at the RT active site provides multiple conformations, which do not experience any unfavorable interaction with the Asp185. Therefore, the DNA primer containing the tenofivir can easily move for the chain elongation, which can prevent excision. (Supported by NIH AI32351)

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Development of TK1 deficiency resulting in cellular resistance against AZT in human T-lymphoid cells is prevented by inhibition of the DNA methyltransferases

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Prolonged treatment of human T-Lymphoid cells (H9) with 3´-Azido-3´-Deoxythymidin (AZT) leads to decreased thymidine kinase 1 expression, which accounts for the failure of AZT to inhibit HIV-1 replication in this cells. A major mechanism in downregulating TK1 expression is the de novo methylation of the TK1 gene promoter region. In the present study we tested wether co-treatment with AZT and methylation inhibitors may prevent the development of AZT resistance. Cells treated for 8 weeks with AZT showed a significant decrease in TK1 expression compared to untreated cells. Cells treated in combination with AZT and the methylation inhibitor 5-azacytidine showed significant higher levels of TK expression compared to cells treated with AZT only. Moreover the antiviral activity of AZT was significantly reduced in cells treated with AZT only, but not in cells treated with AZT and an additional methylation inhibitor. The results suggest that specific inhibition of the de novo DNA-methyltransferases DNMT3a and 3b may provide a novel approach in preventing the development of cellular resistance against NRTIs.

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Effects of HIV Protease Inhibitors (PIs) on Lipid Accumulation and Glucose Uptake in Human Adipocytes. G. Bahador*, G.-X. He, and T. Cihlar. Gilead Sciences, Foster City, CA, USA.

Long-term treatment of HIV-infected patients with PIs is believed to be associated with lipid metabolism disorders (lipodystrophy, hyperlipidemia, hypercholesterolemia) and insulin resistance. Although the mechanism and causative relationship of these adverse effects is not completely understood, a number of *in vitro* studies demonstrated significant effects of several PIs on the differentiation, adipogenesis, and glucose uptake in murine adipocytes derived from the 3T3 cell line. However, only very limited comparative data are available from studies with human adipocytes. We have used primary human preadipocytes isolated from subcutaneous fat tissue (BioWhittaker, Inc.) to evaluate the effects of several approved and investigational PIs on cell differentiation and insulin-stimulated glucose uptake via the GLUT4 transporter. Cell differentiation was determined by measuring the intracellular lipid accumulation using fluorescent staining with Nile Red. Following a 7-day incubation, the tested PIs reduced the lipid accumulation in human adipocytes in the following order of decreasing potency: nelfinavir, ritonavir, saquinavir, lopinavir, amprenavir, indinavir, atazanavir, and TMC-114. EC_{50} values ranged from 3.5 to >20 μM. Under the identical conditions, the murine 3T3 adipocytes produced a lower signal in the Nile Red-based assay than human adipocytes suggesting less lipid accumulation. In fully differentiated human adipocytes, 10 μM ritonavir, indinavir, amprenavir, and atazanavir inhibited the insulin-stimulated glucose uptake by 96, 50, 44, and 34 %, respectively. In the insulin-stimulated mouse adipocytes, the same PIs exhibited slightly lower inhibitory potency towards GLUT4. In conclusion, therapeutic concentrations of PIs caused significant effects on the lipid accumulation and insulin-stimulated glucose uptake in primary human adipocytes, a model that might be more relevant than the murine 3T3-derived adipocytes. Although the relative severity of lipid metabolism disorders is not firmly established among different PIs, in case of some compounds (e.g. atazanavir), the in vitro data appear to correlate with clinical outcome.

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Effect of mycophenolic acid on intracellular dioxolane guanosine triphosphate levels in PBMC

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Amdoxovir (DAPD, (-)-β-D-2,6-diaminopurine dioxolane) is a selective inhibitor of HIV-1 replication *in vitro* and *in vivo.* DAPD is deaminated by adenosine deaminase to yield (-)-β-D-dioxolane guanine (DXG) which is subsequently converted to the corresponding 5'-triphosphate (DXG-TP). DXG-TP is a potent inhibitor of the HIV-RT (K_i = 0.019 μM). Mycophenolic Acid (MPA) inhibits the *de novo* synthesis of guanosine nucleotides by inhibition of inosine monophosphate dehydrogenase (IMPDH). Reduction of intracellular deoxyguanosine triphosphate (dGTP) levels through inhibition of IMPDH may effectively increase the intracellular concentration of DXG-TP, thereby augmenting inhibition of HIV replication. The purpose of these experiments was to evaluate the effects of MPA on the intracellular DXG-TP and dGTP levels in peripheral blood mononuclear cells (PBMC). PBMCs were incubated with various concentrations of DXG with or without 0.25μM of MPA and the intracellular concentration of DXG-TP and dGTP measured by Liquid Chromatography Mass Spectrometry (LC-MS-MS). Results of these experiments showed that the intracellular DXG-TP levels increased in a linear fashion with increasing DXG concentrations (5 to 500 μM). Addition of MPA resulted in an approximate 4-fold increase in DXG-TP levels at all concentrations of DXG tested. Maximum DXG-TP levels were observed following 2 hours of incubation with DXG, followed by a steady decline. In the presence of MPA, peak DXG-TP levels were also observed at 2 hours, however, elevated levels were maintained for up to 8 hours. Intracellular dGTP levels decrease approximately 2-fold in cells incubated with MPA. These results indicate that the increased activity observed *in vitro* with the combination of DXG and MPA is due to increased concentrations of the active compound DXG-TP. The data suggest a role for the use of MPA in combination therapy with amdoxovir for the treatment of HIV.

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The Antiviral Activity of the CXCR4 Antagonist AMD3100 is Independent of the Cytokine-Induced CXCR4/HIV Coreceptor Expression Level

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The chemokinereceptor CXCR4 is the main coreceptor used by T-tropic X4 HIV-1 strains to infect its target T-cells. It has been proven that the CXCR4 expression level in T-cells is strongly upregulated by IL-4, a Th2-type cytokine that is secreted preferentially in HIV-infected patients in a later stage of disease. This results in an enhancement of HIV-1 replication in $CD4^+$ T-lymphocytes. We have now evaluated the potency of the CXCR4 antagonist AMD3100 in PHA/IL-2- versus PHA/IL-4-activated T-cells in order to determine whether the compound has comparable CXCR4-antagonistic and anti-HIV-1 effects under these different cytokine treatments. We analysed the CXCR4 expression level and the dose-dependent inhibition of CXCR4 expression by AMD3100, by monitoring the binding of an anti-CXCR4 mAb (clone 12G5). We also determined SDF-1-induced intracellular calcium signaling and HIV-1 replication in these cells in the absence and presence of AMD3100. The CXCR4 expression level in PHA/IL-4-stimulated cells was much higher than in PHA/IL-2- stimulated cells. However, the potency of the bicyclam AMD3100 to block anti-CXCR4 mAb binding, SDF-1-induced intracellular calcium signaling and X4 HIV-1 replication, remained unchanged. Our data indicate that CXCR4 antagonists such as AMD3100 act independently of the HIV-1 coreceptor expression level. These compounds should therefore be useful in suppressing HIV-1 infection in all stages of the disease.

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Development of a CCR5-tropic HIV-1 Transmission Inhibition Assay for Testing Topical Microbicides. C. Osterling[1], C. Lackman-Smith[1], N. Thompson[1], R. Ptak[1], O. Hartley[2], M. Lewis[1], and B.E. Beer[1], [1]Southern Research Institute, Frederick, Maryland, USA [2]Centre Medical Universitaire, Geneva 1228, Switzerland

Since an effective vaccine has not yet been licensed, the development of topical microbicides to prevent transmission of HIV, and other sexually-transmitted diseases, is of major importance. Among compounds tested, polyanionic molecules (e.g., dextran sulfate) have been shown to have activity in transmission inhibition (TI) of HIV strains that enter host cells via the CXCR4 (X4) coreceptor. However, since most mucosally-transmitted HIV strains utilize the low-charged CCR5 (R5) molecule as a coreceptor, such compounds would not be expected to be effective clinical inhibitors.

Therefore, we developed an R5-tropic HIV TI assay that parallels our existing X4-tropic assay. In the R5 assay, MOLT4.CCR5 cells are chronically infected with molecularly cloned HIV-1 JRCSF and then metabolically arrested with mitomycin C. Next, the cells are used to infect GhostHi5 cells in the presence or absence of the test compound. Dextran sulfate and a variety of other polyanionic compounds, known to be active against X4-tropic HIV strains, were inactive in this assay. In addition, while the R5-specific inhibitors AOP-RANTES and TAK-779 were active against cell-free HIV they were inactive against cell-associated HIV transmission. These results suggest possible differences in the mechanism of viral entry in the two systems and demonstrate the importance of testing topical microbicide candidates against R5-tropic HIV in both cell-free and cell-associated assays.

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The Humanized Anti-CCR5 Antibody PRO 140 Effectively Inhibits HIV-1 Entry Without Inhibiting RANTES-Induced Calcium Mobilization. B O'Hara, JP Gardner, TJ Ketas, BM Sullivan, SI Rosenfield, KA Nagashima, PJ Maddon, WC Olson, Progenics Pharmaceuticals, Inc., Tarrytown, NY, USA

Primary HIV-1 isolates utilize the chemokine receptor CCR5 as a coreceptor, thus making CCR5 a promising target for a new class of viral entry inhibitors. We previously reported that the mouse anti-CCR5 monoclonal antibody (mAb) PRO 140 potently blocks entry by a broad range of R5 isolates (Trkola et al., J. Virol. 75:579, 2001). This antibody has been successfully humanized to permit dosing in man and the humanized antibody is entering Phase 1 testing. To characterize the humanized PRO 140, the antibody was examined for activity against a panel of viruses and using different cell types for infection and was also examined for its effects on human immune cell function in vitro. Viruses studied included a broad range of wild-type and drug-resistant HIV-1 isolates and diverse primary target cell types. The effect of the mAb on chemokine-induced calcium mobilization was studied. The humanized PRO 140 prevented infection by a broad range of HIV-1 isolates. The antibody was effective in diverse and clinically relevant primary target cells, such as T cells, macrophages, and dendritic cells (DCs). The IC90 was about 5 ug/ml in each of these diverse testing situations. The antibody was also effective in blocking infection of T cells in trans by DC-associated virus. Little or no effect was seen on chemokine-induced calcium mobilization at levels giving complete inhibition of virus growth. The humanized anti-CCR5 monoclonal antibody PRO 140 thus broadly and potently inhibits growth of HIV-1 R5 strains but without interfering with chemokine-induced calcium mobilization. These characteristics support the advancement of the humanized antibody into clinical trials.

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Studies of the Intracellular Metabolism of Nucleoside Amino Acid Phosphoramidates Utilizing 31P NMR and LC/MS. Jisook Kim and Carston R. Wagner, Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

3'-Azido-3'-deoxythymidine (AZT) amino acid phosphoramidates were designed as prodrugs of 3'-azido-3'-deoxythymidine monophosphate (AZT-MP) and exhibited anticancer and antiviral activity. Preceding results obtained in our lab, show that AZT amino acid phosphoramidate analogs are able to generate significant amounts of phosphorylated AZT. On the basis of this observation, there are few possible intracellular decomposition pathways: direct conversion of phosphoramidate to AZT-MP through P-N bond cleavage or generation of AZT through P-O bond cleavage. Ultimately for both cases, further phosphorylation of AZT or AZT-MP will lead to the formation of AZT-TP which will act as a substrate or inhibitor of DNA polymerases in infected or transformed cells. In this context, it is critical to identify the major metabolite of AZT amino acid phosphoramidates to understand intracellular metabolism of AZT prodrug analogs, and furthermore correlate the level of metabolite to the corresponding analog in different cell lines. In order to carry out studies, 31P NMR and LC/MS approaches were employed to assess the intracellular metabolic profile of AZT amino acid phosphoramidates analogs in the human T?lymphoblastoid cell line CCRF-CEM (CEM-1.3) and peripheral blood mononuclear cell line (PBMC). Both techniques allow the analysis of intracellular level of the parent prodrug and metabolite. The developed methodology can be used to probe the responses in different cell-lines with various prodrugs and provide insight in elucidating the potential mechanisms of intracellular metabolism and penetration of prodrug analogs.

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Decreased expression of Deoxycytidine Kinase (dCK) in lymphocytes of Human Immunodeficiency Virus type 1 (HIV-1) infected patients under Highly Active Antiretroviral Therapy (HAART) with a detectable virus load.

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Previous *in vitro* studies showed that continuous treatment of cell lines with zidovudine (ddC) or lamivudine (3TC) leads to a decreased expression and activity of dCK. This accounts for an insufficient intracellular activation of the deocycytidine analogs and results in a cellular resistance. In the present study we analyse the influence of the deoxycytidine analogs *in vivo*. dCK gene-expression was investigated in peripheral blood mononuclear cells (PBMC). HIV-1 positive blood samples (n=40) with a detectable virus load were compared to blood samples from HIV-1 negative donors (n=20). The levels of dCK were measured with real time PCR (TaqMan). In 10 HIV-1 positive blood samples (25%) we found dCK levels comparable to the cytidine kinase expression of HIV-1 negative blood donors. Thirty samples (75%) from HIV-1 infected patients showed an expression up to 5-fold less dCK when compared with levels from HIV-1 negative blood samples. The results demonstrate that long-term HAART therapy is associated with decreased dCK expression in high number of patients. Further studies should explore whether a low dCK level accounts for decreased activity of antiretroviral drugs and if dCK gene-expression levels can also be used as therapy markers for disease progression.

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The Tat antagonist neomycin B hexa-arginine conjugate inhibits gp120-induced death of human neuroblastoma cells[a]

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Several AIDS patients develop neurological complications, which are referred to HIV-associated dementia (HAD). The HIV-1 coat glycoprotein gp120 has been proposed as the major etiologic agent for neuronal loss reported post-mortem in the brain of AIDS patients. Chemokine receptors may play a role in gp120-triggered neurotoxicity, both in vitro and in vivo, thus being an intriguing target for developing therapeutic strategies aimed to prevent or reduce neuronal damage occurring during HIV infection. We have previously shown that human CHP100 neuroblastoma cells express CXCR4 and CCR5 chemokine receptors and that interaction between gp120 and these receptors contributes to cytotoxicity elicited by the protein. Molecules other than α- and β-chemokines, able to antagonize gp120 binding to neuronal chemokine receptors, may provide a therapeutic strategy to prevent or reduce neuronal damage elicited by the viral protein. To address this, we have examined the ability of a novel HIV-1 transactivator of transcription (Tat) antagonist, the neomycin B hexa-arginine conjugate NeoR, to act as neuroprotectant. NeoR belongs to a novel family of conjugates of L-arginine with aminoglycosides antibiotics (AAC). NeoR displays anti-HIV activity. It inhibits Tat transactivation and antagonizes Tat extracellular activities, such as increased viral production and induction of CXCR4 expression; Tat and NeoR bind to the same cellular targets. This drug penetrates a variety of cells, including neurons, and accumulates intracellularly; NeoR is able to inhibit replication of T-tropic HIV-1 laboratory isolates. NeoR inhibits HIV binding to cells, probably by blocking the CXCR4 co-receptor. Another important finding is that NeoR also inhibits gp120 binding to PBMC. We found that gp120-triggered death is significantly reduced by NeoR and this protective effect seems related to the ability of NeoR to interact with CXCR4 recceptors. This compound could exert a dual role during HIV infection, in that it could be effective in preventing virus replication in the periphery and in the CNS and in reducing neuronal damage subsequent to virus entry into the CNS. To support the latter hypothesis we have provided evidence indicating that NeoR crosses the blood-brain barrier following systemic administration into mice.

[a]J.Neurochem., (2002, in press)

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A short and efficient route towards enantiomerically pure carbocyclic analogues of thymidine: New substrates for thymidylate kinase

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Nucleoside-based inhibitors of reverse transcriptase (RT) were the first drugs to be used in the chemotherapy of AIDS. After entering the cell, these substances should be activated to their triphosphates by cellular kinases. Thus, for the most extensively used drug, 3'-azido-3'-deoxythymidine (AZT), whereas phosphorylation is facile, the product (AZTMP) is a very poor substrate for the second kinase, thymidylate kinase (TmpK). Due to the steric demand of the azido group in the 3'-position of AZTMP compared to the 3'-hydroxyl group of the natural substrate, 2'-deoxythymidine monophosphate (dTMP), the structure of the enzyme changes, which causes a 200-fold decrease in the rate of phosphorylation. As recognized by molecular modeling the increased flexibility of carbocyclic nucleotides should not cause a structural change of the enzyme. Here, we will present the enantioselective synthesis of carbocyclic analogues of thymidine, as well as the corresponding monophosphates by using a new convergent synthetic strategy. The carbocyclic moiety is coupled to the heterocyclic base to obtain carbocyclic thymidine (*carba*-dT) as a key intermediate for further variations in the 3'-position, e.g. for the carbocyclic AZT. This strategy can also be used for the synthesis of carbocyclic BVDU, a nucleoside analogue known to be antivirally active against HSV-1. Additionally the corresponding *cyclo*Sal-triesters of *carba*-AZT and *carba*-BVDU were synthesized and tested for their antiviral activity.

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Interaction of cholinesterases with *cyclo*Sal nucleotides

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The *cyclo*Sal pronucleotide system has been designed for an intracellular delivery of therapeutically active nucleoside monophosphates. As the *cyclo*Sal concept involves an organophosphate moiety, a chemical reactivity similar to those of known cholinesterase inhibitors can not be excluded. Therefore, investigations concerning a possible interaction of cholinesterases with *cyclo*Sal nucleotides had to be carried out. Two types of cholinesterases are found in humans, the highly specific and physiologically essential acetylcholinesterase (AChE, E.C. 3.1.1.7) and the unspecific butyrylcholinesterase (BChE, E.C. 3.1.1.8) of unknown physiological role. A cholinesterase assay based on the method of Rappaport et al. was established. In experiments using purified human AChE and AChE from *electrophorus electricus* as well as a number of different *cyclo*Sal nucleotides as possible inhibitors, no reduction of cholinesterase activity was observed. In contrast, experiments using human serum as a simple source of BChE showed inhibition of BChE in some cases, but with a strong dependence on the chemical structure of the pronucleotide. A structure-activity relationship (SAR) could be obtained. These results were confirmed by control experiments using purified human BChE. As BChE inhibition would be an unwanted side effect in the possible therapeutic application of *cyclo*Sal nucleotides, ways to avoid it had to be worked out. Therefore, the influence of the stereochemistry at the phosphorous atom was determined, and novel substitution patterns in the aromatic ring were established. The synthesis, characterization and antiviral *in vitro* evaluation of some of these new pronucleotides will also be presented.

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A Publicly Available Database for Compounds Tested Against HIV and Other Viral Pathogens.

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The NIAID **Therapeutics Web site** offers a searchable, publicly available, chemical/biological database, of published information pertaining to compounds tested against HIV and opportunistic infections including TB. We are expanding the literature surveillance to other viral pathogens including those of interest to bio-defense. Intended users of the database include chemists, microbiologists, academic researchers, and those interested in anti-viral drug development. The NIAID Web site, developed and maintained by Cygnus Corp. under contract operated by the Division of AIDS, is available at http://chemdb.niaid.nih.gov. The site uses MDL ISIS software for chemical structures and ChemAxon software to provide structure-search capability. All compounds are currently represented on the Web site as gif images. The Web site is divided into a variety of information categories. The ***Chemical Compound DB*** contains over 82,000 compounds tested against HIV, HIV enzymes, other viruses or opportunistic pathogens. This section can be searched by chemical name or synonym, chemical or therapeutic class, the company that has tested or is developing the compound, CAS#, NSC#, molecular formula or molecular weight. The ***Anti-HIV Cellular DB*** contains over 125,000 lines of in vitro inhibitory data (EC50, CC50, TI, viral resistance, etc.) linked to over 61,000 compounds. The ***Anti-HIV Enzyme DB*** contains over 34,000 lines of data (IC50, Ki, Km, etc.) linked to more than 17,000 compounds. The ***Anti-OI Infection DB*** contains over 110,000 lines of data (enzyme, cellular cytotoxicity, in vitro organism assays, and in vivo animal model efficacy) linked to more than 28,000 compounds about half of this data is related to viral pathogens. The ***Bibliographic DB*** contains over 10,000 references from literature, patents, and meeting abstracts that can be searched either by author, title, journal, patent number or year of publication. The references are linked to the chemical structure, anti-HIV or anti-viral data. The database can also be browsed by chemical or therapeutic class.

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Phosphoramidate Derivatives of Carbocyclic L-d4A Nucleosides and Related Analogues.

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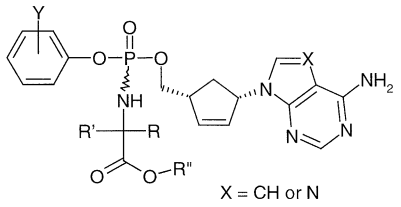
Carbocyclic L-d4A showed modest anti-HBV activity (IC_{50} 1 μM). This prompted us to synthesize phosphoramidate protide derivatives of C-Ld4A and related nucleosides. Potency improvements against HBV of ca. 1000-fold were noted, relative to the C-Ld4A; IC_{50} values for many derivatives were comparable to those of 3TC. These phosphoramidates also showed significantly improved activity against HIV-1. Carbocyclic analogues of D-d4A, 7-deaza-(D- and L-)d4A, and the corresponding phosphoramidates were prepared and evaluated. The SAR relationships for HBV and HIV assays will be compared.



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Unique Mechanism of L-FMAU against Hepatitis B Virus: A Molecular Dynamics Study

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L-FMAU (clevudine) was discovered by our research group as a potent antiviral agent against hepatitis B virus (HBV). However, even though L-FMAU is known to act specifically on viral DNA synthesis, and its triphosphate inhibit the HBV DNA synthesis in a does-dependent manner, the precise understanding of the mechanism of action of L-FMAU at the polymerase level has not been realized. In this study, the recently published homology model of HBV polymerase by Arnold et al. was complexed with L-FMAU-TP and simulated to obtain the structural as well as mechanistic information of L-FMAU-TP binding to the active site of HBV polymerase. Our simulation suggests that the RT-like active site of HBV polymerase was not able to accommodate L-FMAU-TP because docking the L-FMAU-TP at the active site of HBV polymerase caused a steric hindrance. However, during the simulation, the conformational adjustment gave enough space for L-FMAU-TP to undergo the conformational change, which separated the sugar moieties of L-FMAU-TP and 3'-terminal nucleoside of the primer strand from each other. On the other hand, the conformational change in L-FMAU-TP resulted in an interesting change in the polymerization geometry of the growing viral DNA chain. As the polymerization should take place by the S_N2-type reaction of 3'-OH at the α-phosphate moiety, the distance and relative orientation between the two groups are critical. L-FMAU-TP, upon binding to the active site of HBV polymerase, separated additional 2 Å (5.7 Å, the optimum distance is around 3.5 Å.) from the 3'-end of the primer strand due to the conformational change. Therefore, the incorporation of L-FMAU-TP to the growing viral DNA chain is unable to take place due to the deformed polymerization geometry by L-FMAU-TP, which may explain the reason that L-FMAU is not incorporated to the HBV DNA (Supported by NIH AI 32351).

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Dinucleotides as novel class of anti-hepatitis B virus agents: Evaluation of ORI-9020 in a transgenic mouse model. J. G. Julander[a], R. P. Iyer[b], A. Roland[b], Y. Jin[b], , S. Mounir[b], J. D. Morrey[a], [a]Institute for Antiviral Research, Utah State University, Logan, Utah, USA., [b]Spring Bank Technologies, Inc., 100 Barber Avenue, Worcester, MA 01606.

ORI-9020, a novel dinucleotide, was evaluated in transgenic mice expressing hepatitis B virus (HBV). ORI-9020 administered intraperitoneally once daily for 14 days at a dosage of 100 mg/kg/day significantly reduced liver HBV DNA ($P \leq 0.001$). ORI-9020 appeared to indiscriminately remove all HBV DNA species in the Southern blot analysis, including the bands typically left by adefovir dipivoxil (ADV), which may have reflected a different mechanism of action other than classical chain termination. Serum HBe, liver HBs, HBc and RNA were not affected by ORI-9020 treatment, which is consistent in transgenic mice with compounds that block viral synthesis "downstream" from mRNA synthesis, such as blockage of polymerase activities. A minimal effective dosage was determined to be between 1.6 – 0.5 mg/kg/day, which was similar to ADV minimal effective dosage of 1.0 mg/kg/day. Considering the efficacy of ORI-9020 in an HBV animal model and a possible non-chain terminator mechanism of action, this compound should be considered for further analysis. (Supported by Contract NO1-AI-05404, NIAID, NIH)

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Imino sugars with greatly enhanced anti-hepatitis B and BVDV activity and less toxicity than DNJ

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Imino sugar glucosidase inhibitors have selective antiviral activity against certain enveloped, mammalian viruses. Deoxynojirimycins (DNJs) modified by N-alkylation to contain a 9 carbon side chain (N-nonyl-deoxnojirimycin; N-Nonyl-DNJ) were shown to be, for example, at least 40 times more potent in inhibiting hepatitis B virus (HBV) and Bovine Viral Diarrhea Virus (BVDV) in cell based assays than the non-alkylated deoxnojirimycin (DNJ). These data suggested that modification of the alkyl side chain could influence antiviral activity. Previous work has focused upon varying side chain length. In this report, the influence of side chain branching and cyclization upon toxicity and antiviral activity was explored. Briefly, using a virus secretion assay for HBV and a single step growth (yield reduction) assay for BVDV, 14 different deoxynorjirmycin based sugars, possessing various N-alkyl substitutions, were tested for antiviral activity. Of the series, N-oxa-nonyl-DNJ and N-propylcyclohexyl DNJ was determined to have the best selectivity index against BVDV and HBV, with the N-oxa analog being the most potent, with micro molar antiviral activity. The results of this antiviral survey and the implications for the mechanism of action and ultimate therapeutic potential of the deoxynorjirmycin based imino sugars is provided and discussed.

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SeqHepB: A Hepatitis B Virus Sequence Analysis Program for Identifying HBV Mutants Associated with Antiviral Resistance Including Lamivudine, HBIG, Famciclovir, and Adefovir. A Bartholomeusz[1], SA Locarnini[1] A Ayres[1], W Sievert[2], J Sasadeusz[3], P Angus[4] *and Members of the Collaborative Consortium.*
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The SeqHepB system is a web based program comprising a large hepatitis B virus (HBV) genotype database which has been designed to process data from patients with chronic hepatitis B for the purpose of providing support to healthcare professionals. The sequence analysis program has been used to identify unique mutations including those associated with antiviral resistance. Mutations associated with resistance to lamivudine, HBIg, and famciclovir have been previously defined and we have recently identified a single case of resistance to adefovir dipivoxil (ADV). The analysis and early identification of resistance mutations is of importance especially in the treatment of those patients who experience a virological breakthrough on therapy. The SeqHepB genotypic resistance analysis utilizes algorithms of drug susceptibility based on the absence or presence of key mutations identified through genotypic analyses. Key mutations associated with resistance to anti-viral therapy including the ADV resistance mutation have been confirmed with *in vitro* phenotypic assays. The SeqHepB program can also be used to determine the HBV genotype (A-H) and to analyze other clinically important HBV mutations such as the basal core promoter mutations, pre-core stop codon mutations, vaccine escape mutations, or deletions in Pre-S by comparison to reference sequences. Mutations in all overlapping reading frames are detected. Mutations selected by antiviral agents may affect both the polymerase and envelope genes and may alter the immunogenecity and antigen/ antibody binding capacity of the envelope protein. The SeqHepB system is an important tool for individualized patient management and will be a useful guide to antiviral therapy as new antiviral agents and potential combination therapies are introduced, and new HBV resistance mutations are identified.

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Ultrastructural Study of HBV-Producing Cell Lines Cultered in the Absence or Presence of Selective Inhibitors of HBV Replication

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There have been few, if any, reports in which the effect of antivirals on HBV replication is studied at the ultrastructural level. To the best of our knowledge, no electronmicroscopical studies have been carried on the effect of antivirals on HBV-producing cell lines. Here we present an ultrastructural study of the HBV-producing cell line HepAD38, cultured in the absence or presence of selective inhibitors of HBV replication [i.e. adefovir (PMEA) or lamivudine (3TC)]. In the HepAD38 cell line HBV replication is under the control of an inducible tetracycline promoter, which offers the advantage that, unlike the situation with HepG2.2.15 cells, there is no background replication in uninduced cells. In addition, HepAD38 cells produce much larger quantities of viral DNA than HepG2 2.2.15 cells. Treatment with either PMEA or 3TC markedly reduce the total number of viral particles in the cytoplasm. Since the HBV reverse transcriptase/DNA polymerase is believed to convert pregenomic RNA to (partially) double-stranded DNA only after encapsidation has taken place, this is an intriguing observation. In contrast, in the nucleus of many drug-treated cells, an increase in the number of particles was observed. However, the particles in the nucleus were smaller (~28 nm) than those detected in the cytoplasm (35-40 nm) and, on inspection, appeared empty as well. Since we found that viral antigen production (HbsAg) was not reduced in the drug-treated cells as compared to untreated cells, we hypothesize that the nuclear particles consist of self-assembled core proteins that are present in excess (because the core protein is not "consumed" to form viral particles) in the drug-treated cells.

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LB80317, a Novel Anti-HBV Agent, is Very Potent Against Wild Type as well as Lamivudine-resistant HBV Viruses.

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We have discovered a novel phosphonate nucleoside, LB80317, with a potent, selective and specific anti-HBV activities against wild type as well as lamivudine-resistant viruses. The 50% effective concentrations (EC50) for the inhibition of virus replications were measured by using recombinant baculoviruses which encode lamivudine-resistant strains of HBV. EC50 of LB80317 against wild type, MI, MV, LM/MI and LM/MV mutant virus are 0.5 uM, 9.5 uM, 1.0 uM, 2.7 uM and 8.3 uM, respectively. In vivo kidney toxicity is a severe problem for some antiviral nucleoside analogues. The renal uptake by human Renal Organic Anion Transporter 1 (hOAT1) plays a critical role in kidney-specific toxicity of phosphonate nucleotide derivatives. To compare and estimate the kidney toxicity of LB80317 and its derivatives with adefovir, in vitro kidney cytotoxicity model was developed. Human hOAT1 cDNA from kidney tissue was cloned by RT-PCR. The cDNA was confirmed by sequencing. Transient and stable expression of hOAT cDNA showed probenecid-sensitive uptakes of p-aminohippurate and cytotoxicity to adefovir. In chinese hamster ovary cells, the stable expression of hOAT1 showed approximately 200-fold more toxicity compared to parental non-expression cell. In comparison of cytotoxicity using stable hOAT1-expression cell lines, LB80317 and its derivatives showed approximately 50-fold less toxicity than adefovir. LB80380, a prodrug of LB80317, was evaluated for antiviral effect in vivo both in HBV-producing transgenic mice and woodchucks. LB80380 is in clinical phase II trials for the treatment of chronic human HBV infections.

Oral Session III: Herpesiruses

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A Thymidine Phosphorylase-Stable Analog of BVDU with Significant Antiviral Activity

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Nucleoside analogs, which target viral DNA polymerase, represent an important class of anti-HSV drugs. These inhibitors require phosphorylation by HSV thymidine kinase (TK) to the respective deoxynucleoside triphosphate (dNTP) to express their activity. (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) is a potent and selective antiviral agent against HSV-1 and VZV. However, BVDU is a good substrate for the human pyrimidine nucleoside phosphorylases (PyNP), such as thymidine phosphorylase (TPase). The possible degradation of BVDU by PyNP before its conversion to the triphosphate may be a significant limitation of its antiviral potential. Molecular modeling studies led us to the design and synthesis of an isomeric analog of BVDU in which the nucleobase is translocated from the natural 1'-position to the isomeric 2'-position. (*E*)-5-(2-Bromovinyl)isodideoxyuridine (BVisoDDU) is selectively active against HSV-1 (three different strains) but inactive against HSV-2. Unlike BVDU, BVisoDDU is completely resistant to cleavage by thymidine phosphorylase. BVisoDDU is also the first nucleoside analog lacking OH groups at both the 2'- and 3'-position that shows pronounced activity against HSV-1 replication.

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Discovery of a new family of Bicyclic Dideoxy Nucleosides which Inhibit HCMV by a non-nucleoside mechanism.

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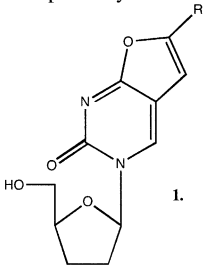
As part of our programme of lead optimisation of our newly discovered highly potent anti-VZV and evaluated some novel 2',3'-dideoxy analogues, such as (1). These compounds, prepared via Pd-catalysed coupling of terminal alkynes with 5-iodo-2',3'-deoxyuridine, have only limited anti-VZV activity, but emerge as potent and selective anti-HCMV agents. As with parent anti-VZV agents, the present compounds have an absolute dependence on an alkyl side chain for antiviral activity. Lastly, and surprisingly, MoA studies reveal these 'nucleosides' to act via a non-nucleoside mechanism, early in viral replication and probably at viral fusion.



1.

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In Vitro Anti-Herpesvirus Activity of 4-Hydroxyquinoline Carboxamide (4-HQC) Analogs, A Novel Class of Non-Nucleoside Broad-Spectrum Polymerase Inhibitors.

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We have reported previously that PHA-183792, a 4-oxo-dihydroquinoline inhibited the replication of herpes simplex virus type 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HCMV), varicella zoster virus (VZV, and Epstein-Barr Virus (EBV). Importantly, the compound also retained activity against HSV and VZV isolates resistant to Acyclovir (ACV) and HCMV strains resistant to ganciclovir (GCV). We have now extended these studies to include three additional 4-HQC analogues as well as evaluation against human herpes viruses types 6A, 6B, and 8 (HHV-6A, HHV-6B, and HHV-8). The prototype PHA-183792 was active against HSV, CMV, VZV, EBV, and HHV-8 at concentrations of 1-2 μg/ml which was equivalent to ACV or GCV. None of the compounds were active against either HHV-6 strain in the assays used in these studies. Of the analogues tested, PHA-529311 was clearly more active than PHA-183792, ACV or GCV; the activity of PHA-570886 was equivalent, whereas PHA-568561 was less active than PHA-183792, ACV or GCV. All the 4-HQC analogs retained their activity against HSV-1, HSV-2, CMV, or VZV isolates that were resistant to ACV or GCV and CMV isolates that were resistant to foscarnet or cidofovir. In summary; 1) selected analogues of this class of compounds inhibited the replication of all the herpesviruses tested except HHV-6; 2) were active against all the drug resistant mutants tested and; 3) may provide an alternative treatment for herpesvirus infections, particularly those resistant to current therapies.

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The antiviral activity of CTC-8 a member of novel class of herpesvirus selective inhibitors based on prostaglandin analogues with a putative cellular target for the inhibition of herpes viruses.

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Prostaglandin-A_1 was reported to be a specific inhibitor of HSV-1 [*M.G. Santoro, Trends Microbiol.,5,276-281,1997;* review]. This provided the rationale for a synthetic programme of derivative compounds with potential anti-inflammatory and/or antiviral properties. The compound CTC-8 (structure to be shown) is one of a series of cyclopentenone-based compounds with biological activity. The addition of CTC-8 to HEp-2 or BHK cells infected with HSV-1 showed a significant decrease in virus yield at 24h (ED90 <1mM). Both cell-associated and released virus were reduced and the antiviral effect was similar under conditions of low and high multiplicity of infection. However, no antiviral activity was detected using a conventional plaque reduction assay. Cytotoxicity in both resting and dividing cells was observed at higher drug concentrations (CC50 >10mM) using a quantitative viability assay indicating a modest selective index. Interestingly, time-of-addition studies showed that the antiviral activity was lost when the compound was added 24h before infection and remained present. Conversely, the greatest antiviral effect was seen when the addition of compound was delayed to 6-12h post infection when reductions in yield of up to 3 log10 were recorded. Extended passage of virus in the presence of CTC-8 produced no selection of resistance to the compound. The evidence in favour of a cellular target for antiviral activity will be discussed and, based on these mechanistic studies, the utility of this and similar compounds as potential therapeutic agents, either alone or in combination, will be considered.

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The IκB kinase IKK is the molecular target for anti-herpetic cyclopentenones.

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The IκB kinase (IKK) complex is a key enzyme in signaling the activation of nuclear factor-κB (NF-κB), a critical regulator of the innate response to pathogens mediating inducible expression of multiple genes involved in inflammation and immunity. We have recently shown that HSV type 1 (HSV-1) is a potent inducer of NF-κB persistent activation in human cells and mimic pro-inflammatory cytokines utilizing a cellular signaling pathway involving IKK stimulation. IKK activity induced in the early phase of infection is followed by phosphorylation and degradation of the inhibitory protein IκBα and by transcriptional activation of NF-κB-dependent cellular and viral genes. IKK inhibition by anti-inflammatory cyclopentenone prostanoids (cyPG) blocks HSV-1 gene expression and reduces virus yield by more than 3000-fold. We have identified the β subunit of IKK as the molecular target of cyPG. Analysis of the molecular structure of cyPG has identified the cyclopentenone ring (2-cyclopenten-1-one, 2-Cy) as the active moiety. Structure-activity relationship studies on a variety of 2-Cy derivatives indicate that minor differences in the side chains translate to significant differences in NF-κB inhibitory potency and in antiviral activity. The results identify IKK as a potential target for anti-herpetic drugs and stimulated the development of a new class of cyclopentenone derivatives for the treatment of herpesvirus infection.

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Evaluation of a Recombinant Live Attenuated Herpes Simplex Virus Type 2 Vaccine in Guinea Pigs.

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Recombinant virus AD472 was constructed by deleting both copies of the $\gamma_1$34.5 gene, as well as the UL43.5, UL55-56 and US11-12 open reading frames from HSV-2 (G). Both copies of ORF P have also been deleted because they reside on the opposite strand of the viral DNA as the $\gamma_1$34.5 gene. The thymidine kinase gene remains functional and confers susceptibility to acyclovir. The resulting recombinant virus replicates well in MRC-5 cells, is genotypically stable and exhibits the expected phenotype of neuroattenuation. Serial passage of this vaccine candidate through the CNS of susceptible mice demonstrated that this phenotype was stable following 9 serial passages. The potential use of this virus as an HSV-2 vaccine candidate was investigated by evaluating the ability of this virus to protect guinea pigs from severe genital herpes by HSV-2 (G) challenge. The results indicate that AD472 administered intramuscularly was effective at reducing both lesion development and severity in guinea pigs infected intravaginally with HSV-2. The positive outcome of these studies offers encouragement that it is possible to produce and manufacture a safe and effective live attenuated HSV-2 vaccine.

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A New Antiviral Mechanism: Mutation of Human Cytomegalovirus Gene UL27 Confers Resistance to the Antiviral Drug 1263W94. G. Komazin, R. G. Ptak, B. T. Emmer, D. L. Evers, L. B. Townsend, and J. C. Drach. School of Dentistry and College of Pharmacy, University of Michigan, Ann Arbor, Michigan U.S.A.

We and our collaborators have previously reported that the activity of D- and L-ribosyl benzimidazoles TCRB and 1263W94 against human cytomegalovirus (HCMV) involves gene products UL56 plus UL89 and UL97, respectively. To further investigate mode of action of these compounds we have isolated an HCMV strain termed G2, that is resistant to both TCRB and 1263W94. HCMV Towne strain C4 (with the UL56 and UL89 mutations) was passaged in increasing concentrations of 1263W94, up to 64 μM. The resulting isolate, termed G2 was purified by the Klein method. It was ~20-fold resistant by yield assay and grew at the same rate as Towne. Genotypic characterization of this strain confirmed the presence of the expected mutations in UL56 and UL89 genes. No mutations which could explain resistance to 1263W94 were found in gene UL97 nor in other genes involved in DNA synthesis and packaging (UL44, UL57, UL98, UL105, UL37ex1, UL104, UL77, UL51, UL52, UL93). A cosmid library of the G2 HCMV genome was constructed. Regions of an HCMV bacterial artificial chromosome corresponding to the G2 cosmids were deleted and combined with selected cosmids to construct recombinant viruses. Drug susceptibility by plaque assay narrowed the location of the mutation to open reading frames UL26-UL31. These genes were sequenced and a mutation in gene UL27 implying leu335pro was found to be responsible for resistance to 1263W94. We conclude that the product of HCMV gene UL27 is involved in the antiviral activity of 1263W94 based upon the observation that a single putative amino acid change in this gene was necessary and sufficient to produce drug resistance. These studies were supported by N.I.H. grants U19-AI317I8 and PO1-AI46390.

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CMV423, a potent and selective inhibitor of human herpesvirus 6 exerts its antiviral action through inhibition of cellular protein tyrosine kinase activity

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CMV423 (2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine -1-carboxamide) is a new antiviral agent with potent and selective *in vitro* activity against the β-herpesvirus human cytomegalovirus (HCMV), but not against α- or γ-herpesviruses [Snoeck *et al.* (2002), Antiviral Res. **55**: 413-424]. Here we report that its activity also extends to human herpesvirus 6 (HHV-6) and 7 (HHV-7). When compared *in vitro* to ganciclovir and foscarnet (the standard drugs recommended in the treatment of HHV-6 infections), CMV423 showed a superior selectivity, due to its high activity (IC_{50}: 53 nM) and low cytotoxicity (CC_{50}: 144 μM), both in continuous cell lines and in primary peripheral blood lymphocytes infected with HHV-6. From mechanistic experiments at the level of viral mRNA and protein expression, we learned that CMV423 targets an event following viral entry but preceding viral DNA replication. Its antiviral action was dependent on the cell line used, implying involvement of a cellular component. We demonstrated that CMV423 has an inhibitory effect on the total cellular protein tyrosine kinase activity, in accordance with the synergistic anti-HHV-6 activity of CMV423 when combined with the tyrosine kinase inhibitor herbimycin A. The activities of cyclin-dependent kinases, protein kinase A and C, and the HHV-6-encoded pU69 kinase were not affected. We therefore conclude that CMV423 exerts its activity against HHV-6 through an inhibition of cellular protein tyrosine kinase activity.

Oral Session IV: Minisymposium – Perspectives in Biodefense, How Prepared Are We?

No Abstracts

Poster Session II: Herpesviruses, Poxviruses

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Inhibition of Herpesvirus Replication in Vitro by a Series of 4-Oxo-hydroquinoline Carboxamides with Viral Polymerase Activity.

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Herpesviruses cause a wide variety of human diseases ranging from cold sores and genital herpes to encephalitis, congenital infections and lymphoproliferative diseases. These opportunistic viruses cause major problems in immunocompromised individuals such as transplant recipients, cancer patients, and HIV infected persons. The current treatment of these infections with nucleoside analogs such as acyclovir (ACV), ganciclovir (GCV), or cidofovir (CDV) has improved the outcome for many but long term treatment has problems with toxicity as well as the emergence of resistant viruses. Therefore, there is a need for new compounds that might be used in place of or in addition to the current therapies. We have evaluated a new series of compounds which have a different mechanism of action than nucleosides and have activity against Herpes Simplex virus (HSV), Cytomegalovirus (CMV), Varicella Zoster (VZV), Epstein Barr virus (EBV) and Herpesvirus type 8 (HHV-8), but not Herpesvirus type 6 (HHV-6). Of the four compounds evaluated, two (PHA-529311 and PHA-570886) had equal or greater activity than the parent, PHA-183792, against multiple virus strains and one (PHA-568561) was less effective than the parent. These compounds had greater efficacy against CMV than other herpesviruses tested (PHA-183792 2.1μg/ml, PHA-529311 0.5μg/ml, PHA-568561 2.4μg/ml and PHA-570886 0.7μg/ml). These new analogs also had as good as, if not greater activity than the parent compound against ACV-resistant HSV and VZV isolates and GCV or PFA-resistant CMV isolates. These results confirm the broad-spectrum efficacy of these compounds against multiple members of the herpesvirus group and suggest that members of this class may have a potential role for treatment of a variety of herpesvirus infections.

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In Vitro Activity of Alkoxyalkyl Esters of Cidofovir against replication of Herpesviruses.

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Previously, we have described the activity of the cidofovir (CDV) analogues, hexadecyloxypropyl-CDV (HDP-CDV) and octadecyloxyethyl-CDV (ODE-CDV) against herpesvirus replication in cell culture. These compounds demonstrated significantly greater activity than CDV against all the herpesviruses tested. In an effort to evaluate structure-activity relationships in this series of compounds, additional alkoxyalkyl esters were synthesized including octadecyloxy-propyl-CDV (ODP-CDV), oleyloxyethyl-CDV (OLE-CDV), and oleyloxypropyl-CDV (OLP-CDV). We evaluated the in vitro activity of these compounds against human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), herpes simplex virus types 1 and 2 (HSV-1 and 2), Varicella-Zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus type 6 (HHV-6), and human herpesvirus type 8 (HHV-8). In plaque reduction assays, EC_{50} values for ODP-CDV, OLE-CDV, and OLP-CDV against HCMV were 0.002, 0.002 and 0.002μM, respectively. In addition, similar EC_{50} values were observed against MCMV. An EC_{50} value of 0.02μM was obtained for the three analogs versus HSV-1 and EC_{50} values were about ten-fold or 0.002μM against HSV-2. Using an Elisa assay to measure EBV replication, EC_{50} values of 0.8, <0.003 and 0.05μM were obtained for ODP-CDV, OLE-CDV, and OLP-CDV respectively. In most of the assays performed, the analogs had EC_{50} values that were at least a hundred fold lower than the EC_{50} values for CDV which ranged from 0.3 to 59μM. These results indicate that these alkoxyalkyl CDV analogs are all considerably more active than CDV itself against all the viruses tested and suggest that they may have potential for treatment of infections caused by the herpesviruses.

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Bicyclic Furo Pyrimidine Nucleosides with Haloaryl Side Chains.

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Since our discovery of bicyclic furo pyrimidine analogues as potent and selective inhibitors of Varicella Zoster Virus (VZV), several studies have been made to investigate the structure-activity relationships (SAR's) of these compounds and to seek to increase antiviral activity. The most potent compounds reported to date are *p*-alkylphenyl derivatives with EC_{50} values below 1 nanomolar versus VZV and selectivity index values of *c.a.* 1,000,000. In further explorations in this work, we report the synthesis and biological evaluation of haloaryl derivatives. The target structures were prepared in a one-pot synthesis by the Pd(0)-catalysed coupling of the appropriate haloaryl derivatives with 5-ethynyl-2'-deoxyuridine to give the 5-alkynyl nucleosides which were subsequently cyclised in the presence of CuI and Et_3N. 5-Ethynyl-2'-deoxyuridine was synthesised by coupling 5-iodo-2'-deoxyuridine with trimethylsilylacetylene followed by desilylation in the presence of NH_3. The initial biological information of mono substitutions shows that, substitution at the para position retained antiviral activity (with the exception of fluoro), meta substitution shows a decrease in antiviral activity and ortho substitution of a halo atom shows an increase in antiviral activity against VZV. Further Data will be presented at the meeting.

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Combined AntiHSV-1 Activity of New Dimeric Derivatives of Netropsin with modified nucleosides and phosphonoacetic acid (PAA) in Vero cells.

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The new derivatives of netropsin (I) are bis-netropsins in which two monomers are linked in a tail-to-tail (II) or head-to-tail (III) manner. The compounds II and III selectively interact with viral DNA. Unlike I the bis-netropsins II and III have equal highl selective antiherpes activity (ID_{50}=5μg/ml; IS=60 and 35, respectively), including strains of HSV-1 resistant to acyclovir (ACV) and PAA. Synergistic antiHSV-1 effect of combinations of II and III with ACV, ganciclovir, BVDU, AraA and PAA was obtained. If combinations II, III and nucleoside analogues or PAA are used the concentrations of II and III are reduced in 15-60 times; in these conditions concentrations of the nucleoside analogues and PAA can be reduced in 4-9 times. For example, ID_{50}(ACV) = 0,45 μg/ml; ID_{50} (ACV + II) = 0,1 μg/ml + 0,15 μg/ml. On model of vaccinia virus (VV) the values of ID_{50} of II and III are equalized 1,5 and 3,0 μg/ml, respectively. Combined use of II + AraA proved with synergistic antiVV effect. CONCLUSION: Combined use of modified nucleosides or PAA with II and III permits increase selectivity of antiviral action.

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Antiherpetic Activity of Some 5-arylethynyl Derivatives of 2'-deoxyuridine in Vero Cells.

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AntiHSV-1 action of new derivatives of 2'-deoxyuridine was studied. Antiviral activity of compounds was evaluated on its ability to inhibited virus-induced CPE in cells infected by HSV-1 including three strains resistant to acyclovit (ACV) (ID_{50} > 100 μg/ml), trisodiumphosphonoformate (PFA) (ID_{50} = 120 μg/ml) and its combination (ID_{50} > 100 and 120 μg/ml, respectively). 5-(3-perenyleneethynyl)-2'-deoxyuridine (I) inhibited reproduction of all tested strains of HSV-1 (CD_{50}=250 μg/ml; ID_{50}=7,8-31,2 μg/ml; ID_{95}=15,6-62,5 μg/ml). Compound II 5-(1-pyrenyeethylnyl)-2'-deoxyuridine inhibited reproduction of all strains equally (CD_{50} > 125 μg/ml; ID_{50}=15,6 μg/ml; ID_{95}=31,2-62,5 μg/ml. 5-[4-(2-benzoxazolyl)phenylethynyl]-2'-deoxyuridine (III) inhibited reproduction of ACV-sensitive strain of HSV-1 (CD_{50}=62,5 μg/ml; ID_{50}=7,8 μg/ml; ID_{95}=15,6 μg/ml). III don't inhibit reproduction of strains resistant to ACV, PFA (ID_{50}=31,2-62,5 μg/ml; $ID_{95} \geq$ 62,5 μg/ml). I, II, III have not any activity on model of Sindbis virus (RNA-contented virus) and vaccinia virus in Vero cells. These dates indicated on specificity of antiherpetic effect of I, II, III.

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Dynamics of *N*-Methanocarbathymidine Antiherpetic Activity

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N-Methanocarbathymidine, (N)-MCT), a thymidine analog incorporating a pseudosugar with a fixed Northern conformation, exhibits potent antiherpetic activity against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2). (N)-MCT showed potency greater than ganciclovir (GCV)--the IC_{50}s were 0.02 μM and 0.25 μM for (N)-MCT and GCV, respectively. The standard plaque reduction assay to measure viral infection was used. Treatment of Vero cells infected with 1 m.o.i of HSV-1 with (N)-MCT immediately post-infection (p.i.) or up till 10 h. p.i. profoundly inhibited the development of HSV-1 infection. However, when the infected Vero cells where treated with (N)-MCT at 18 h p.i. or later, there was no inhibitory effect on the development of the viral infection. When Vero cell culture was partially infected with 0.1 m.o.i. of HSV-1 and then treated with (N)-MCT, even up till two days p.i. there was a significant inhibition of viral infection. Our results showed that one dose of (N)-MCT was sufficient for full prevention of viral infection development when applied no later than 10 h. p.i. and that continuous treatment with this chemical was not required. This could be explained by the fact that the majority of the viral DNA synthesis in HSV-1 infected cells occurs during the first 10 h p.i. (N)-MCT showed no cytotoxic effect on noninfected Vero cells (CC_{50} > 100 μM). These results are in good agreement with data we obtained from the analysis of the metabolic pathway of (N)-MCT in HSV-1 infected cells. These data showed high levels of (N)-MCT-triphosphate [(N)-MCT-TP] after 1 h of treatment of HSV-1-infected cells with (N)-MCT. In contrast, noninfected cells incubated with (N)-MCT showed elevated levels of (N)-MCT-monophosphate only. These results suggest that the antiviral activity of (N)-MCT against herpes viruses is mediated through its triphosphate metabolite.

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Antiviral Activity of Triplex-Forming Oligonucleotides Against HSV-1

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The currently available antiviral agents active against herpesviruses require ongoing viral replication. Therefore, nucleic acid analogs such as acyclovir have no activity against latent herpes simplex viruses (HSV). We used the "antigene" or "triplex-forming oligonucleotide" (TFO) approach to design antiviral oligonucleotides that may be active against latent HSV-1. TFOs form a third DNA strand --the triplex-- that is bound non-covalently to specific target sequences to induce mutations or affect transcription. Two target sequences 20 and 24 bp in length were identified within the HSV-1 latency associated transcripts (LAT) domain. Phosphodiester-backboned, purine motif TFOs (TFO1 and TFO3) were synthesized along with matching scrambled, control oligonucleotides. Triplex formation in vitro was shown by incubating increasing concentrations of TFO3 with its HSV-1 LAT target site. ^{32}P-labeled TFO3 placed into the medium bathing primary rat neuron cell cultures was concentrated in the cells approximately 100-fold after 24 hours. One micromolar TFO3 significantly inhibited luciferase production when added to HEK-293 cells transfected with an HSV-1 target-containing reporter construct. Addition of 1.0 μM TFO3 to HSV-1 in vitro followed by 40 minutes of ultraviolet-A light activation gave a 70% plaque-reduction effect compared with virus alone or virus + control TFO3 ($p<.001$). The TFO-induced antiviral effects were specific and reproducible under physiologic conditions. One micromolar TFO1 by itself had no measured antiviral effect. However, 1.0 μM TFO1 in combination with 1.0 μM TFO3 provided a synergistic antiviral effect giving a 1.0-log plaque-reduction compared with the control TFOs ($p<.01$). These studies offer the prospect of a novel antiviral agent that may have activity against latent HSV-1.

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Metalloporphyrins as Agents Against HSV

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We have found that porphyrins are active against HSV. The addition of a metal to the central core can change a number of properties of the porphyrin including solubility and self-stacking. Metalloporphyrins can also have altered interactions with proteins due to one of the amino acids in the protein serving as an axial ligand for the metal, or, conversely, due to axial ligands on the metal interfering with protein binding. Herein, we report the inhibition of HSV-1 and HSV-2 by more than 100 metalloporphyrins and the corresponding free base porphyrins. Selected members of both the natural porphyrins (e.g., resembling hemin) and synthetic (e.g., based on tetraphenylporphyrin) show significant inhibition of the virus at 1 ug/mL. A few examples show significantly different inhibition of HSV-1 in comparison to HSV-2. Some paramagnetic chelates have very high activity (e.g., Cu and Fe). Paramagnetic chelates are not photoactive, a desirable feature in that photoactivity might lead to toxicity in extended use.

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Specific inhibition of virus replication by binding of a *N,N'*-bisheteryl derivative of dispirotripiperazine (DSTP) to heparan sulfate (HS) residues on the cell surface

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The molecular mechanism of the inhibitory activity of DSTP 27, a representative of a new class of antiherpetic compounds with dispirotripiperazine structure (Schmidtke et al. Antivir. Res. 2002), was investigated. DSTP 27 has no virucidal effect on virus particles but inhibits virus attachment by blockade of cellular virus receptors. Preincubation of target cells led to drastic reduction of HSV-1 replication. The virus receptors involved were identified as HS containing cellular structures by 3 lines of evidence: (1) *In vitro* binding of DSTP 27 to heparin acrylic beads was shoen. (2) The growth of gC-positive but not gC-negative pseudorabies virus mutants (PrV) was inhibited by DSTP-27. (3) Inhibition of the gC-positive PrV was only achieved in L cells but not in HS-negative L cells mutants. These results demonstrate that the inhibitory activity of DSTP 27 on viral growth requires the presence of HS on the cell surface and a HS binding viral attachment protein. In addition, cell protection persisted up to 4 hours after removal of the inhibitor. DSTP 27 also efficiently inhibits a number of other HSV-1 and HSV-2 strains including acyclovir- and foscarnet-resistant strains, HCMV, HRSV, and some retroviruses using HS as receptor or coreceptor.

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The synergistic effects of betulin with acyclovir against herpes simplex viruses Yunhao Gong[a], Carolyn Luscombe[b], Izabelle Gadawski[a], Dorothy Cheung[a], Teresa Tam[a], Stephen Sacks[a,c] a. Viridae Clinical Sciences Inc., Vancouver, British Columbia, Canada; b. NaturTek, LLC of 5013 Miller Trunk Highway, Duluth, Minnesota, USA; c. Department of Pharmacology and Therapeutics, The University of British Columbia, Vancouver, BC, Canada

Betulin, a pentacyclic triterpenoid, was isolated from the bark of *Betula papyrifera* by Dr P. Krasutsky of University of Minnesota in Duluth. The University of Minnesota in Duluth investigated the antiviral efficacies of betulin on herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) (patents: US5,750,578/1998, US 6,369,101/2002). HSV (type 1 and 2) infections were evaluated using viral plaque reduction assays on Vero cells. The results indicate that betulin is active against both HSV-1 and HSV-2 infections with the 50% effective concentrations (EC_{50}) of 0.4 μg/ml and 4.2 μg/ml, respectively. The cytotoxicity of betulin was examined on Vero cells using neutral red uptake assay. The 50% cytotoxic concentration (CC_{50}) of betulin was 73.1 μg/ml.

The synergistic effects of betulin with acyclovir (ACV) on HSV-1 and HSV-2 infections were initially examined by Keyel et al. (2000) (The National Conference on Undergraduate Research). We confirmed this observation by incubation of varying concentrations of betulin compound with various concentrations of ACV in fixed ratios using plaque reduction assays. The combination indexes were then analyzed by median-effect equation of Chou. Synergy and moderate synergy were observed for betulin and ACV on HSV-1 when the concentrations of ACV and betulin were higher than 0.068 and 0.4 μg/ml, respectively. At the lower concentrations, additive effect was seen. Synergistic effect was also observed on HSV-2 at higher concentrations, i.e. 0.45 μg/ml of ACV combined with 8.4 μg/ml of betulin. At lower concentrations of ACV and betulin, additive effect, and even moderate antagonistic effect were examined. Further studies to determine the potential mode of action for betulin as treatments for HSV infections are warranted.

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Establishment of herpes simplex virus (HSV) latency model using PC12 cells and the antiviral activity of pencyclovir on HSV reactivation Yunhao Gong[a], John Meligeni[b], Ying-Hsiu Su[c], Teresa Tam[a], Izabelle Gadawski[a], Stephen Sacks[a,d] a. Viridae Clinical Sciences Inc., Vancouver, Canada; b. Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA; c. Jefferson Center for Biomedical Research, Doylestown, Pennsylvania; d. Department of Pharmacology & Therapeutics, The University of British Columbia, Vancouver.

HSV latent infection and the suppression of HSV reactivation by pencyclovir (PCV) were investigated in neurodifferentiated, rat pheochromocytoma cells (PC-12). PC-12 cells were differentiated into neuronal forms by incubation with nerve growth factor. Undifferentiated cells were removed by incubation with 5-fluorodeoxyuridine. The purified neurodifferentiated PC-12 cells were incubated with HSV-1 or HSV-2 to establish infection. Latent infection was demonstrated at 8 days after infection, when 1-3 copies of latency-associated transcripts per cell were measured by real time PCR, in the absence of infectious virions in the culture medium measured by standard plaque assay.

HSV-1 or HSV-2 replication was stimulated in latently infected, neurodifferentiated PC-12 cells by co-cultivation with mammalian cells. Reactivation of HSV-1 and HSV-2 replication was incomplete in that quantitative assessments of the intracellular HSV content did not reach the same level as that assayed immediately after infection. HSV-1 reactivation was measured when latently infected, neurodifferentiated PC-12 cells had been co-cultured with HepG2 cells. HSV-2 reactivation was measured when latently infected, neurodifferentiated PC-12 cells had been co-cultured with HepG2, Vero, or MRC-5 cells.

PCV blocked reactivation of HSV-1 or HSV-2. Uptake of PCV from the culture media into latently infected, neurodifferentiated PC-12 cells was demonstrated by microcapillary electrophoresis. Incubation of latently infected, neurodifferentiated PC-12 cells with PCV during co-cultivation blocked replication of both HSV-1 and HSV-2. The concentration of PCV that blocked replication of HSV-1 in half of the co-cultures was < 0.3 µg/ml. The concentration of PCV that blocked replication of HSV-2 in half of the co-cultures was ~ 0.5 µg/ml.

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Construction of an Expression Vector Encoding Glycoprotein D of Herpes Simplex Virus Type-1 for Detection of the Expressed Protein by Fluorescent Technique.

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Herpes simplex virus type-1 (HSV-1) infection constitutes a serious worldwide public health problem. There are 11 known antigenically distinct glycoproteins in HSV-1 virion. Glycoprotein D (gD-1) is one of the most important target and primary inducers of humoral immune responses in HSV-1 infected hosts. In order to construct a plasmid expression vector capable of expressing gD-1 in an eukaryotic system a 1.2 kb BamH1 DNA fragment containing full-length of gD-1 gene was cloned into the PcDNA3 expression vector. Digesting the clone by Nar-1 restriction enzyme, and analyzing the resultant electrophoretically determined the orientation of the inserted gene. Clones carrying the gene of interest with right orientation were purified and transfected to Cos-7 cells by liposome. Expression of the protein in the transfected cells was confirmed by the immunofluorescence technique.

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Construction of an Expression Vector Containing Main Neutralizing Epitopes of Herpes Simplex Virus Type 1 Glycoprotein B and Its Expression in Mammalian Cell Lines.

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One of the most important immunogenic proteins of herpes simplex type 1(HSV-1) is glycoprotein B (gB-1). The first 1520 nucleotides of gB-1 gene contain the main neutralizing epitopes and is likely to be used instead of gB-1 gene for inducing the humoral immunity. To study this hypothesis it is needed to construct an expression vector that can be expressed in mammalian cell lines. A clone carrying full length of gB-1 gene was digested with BamH1 and Pst1 to separate the first 1520 nucleotides of the gene. An especial adaptor having 5' overhang of Pst1 and 5' overhang of Xba1, as well as, a stop codon along it, was synthesized. Ligation was then done among the target gene, the adaptor, and the digested expression vector, simultaneusely. The construct was digested with an appropriate restriction enzyme to confirm the exact ligation of the fragments. A dot blot hybridization was also done to reconfirm the result. Cos-7 cells were transfected with construct and the expression of the protein of interest wasdetected using immunofleurescence technique. Although the recombinant protein produced in this study is a secretoty one, we were able to detect that during the first few houres to 40 hour post transfection in the cytoplasm of the cells.

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Application of Recombinant Glycoprotein G and D (gG-1 and gD-1) of Herpes Simplex Virus Type 1 (HSV-1) for Detection of Specific Antibodies.

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Glycoprotein G of herpes simplex virus type-1 was prepared as a recombinant protein in prokaryotic and Eukaryotic systems. E.coli DH5α and buculovirus expression system were used to prepare the above products, respectively. An indirect ELISA method using purified form of the recombinant gG-1 was developed for detection of antibodies against HSV-1 and the result was compared with those obtained from virus neutralization test (VNT). Taking VNT as a gold standard test it was shown that although the sensitivity of ELISA with either recombinant gG-1 was 100% but the specificities of the tests were 89.5% and 94.7% for the protein produced in prokaryotic and Eukaryotic systems, respectively. Glycoprotein D-1 was also produced in eukaryotic system and used to developed a western blotting (WB) test for detection of HSV-1 antibodies. Although there was an antigenic cross reaction between HSV-1 and HSV-2, with regard to the WB using the recombinant gD-1, but gG-1 showed no reaction with serum samples containing only HSV-2 antibodies.

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Molecular Analysis of Clinical Isolates of ACV-Resistant Herpes Simples Virus

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Herpes simplex virus (HSV) isolates resistant to the nucleoside analogue acyclovir (ACV) have been studied genotypically and phenotypically using laboratory derived strains and clinically resistant isolates. The studies have identified various 'hot spots' on the thymidine kinase (TK) gene that confer resistance to ACV. The majority of these are frameshift mutations, namely insertions or deletions at homopolymer nucleotide stretches of guanines (Gs) and cytosines (Cs). Others include random nucleotide substitutions in those regions of the TK gene that appear to be important in enzyme function. Since 1990 we have isolated HSV from patients showing clinical resistance to ACV. We have characterised the antiviral phenotype (ACV resistant (r) or sensitive (s)) and genotype of a collection of 54 clinical herpes isolates: HSV-1 ACVr (n=18), HSV-2 ACVr (n=22), HSV-1 ACVs (n=7) and HSV-2 ACVs (n=7). Our results confirm recognised mutational 'hot spots' as being major determinants of TK-associated ACV resistance. We also identify new polymorphisms associated with heterogeneity of the TK gene but not ACV resistance, and show that several previously undescribed mutations appear to be directly responsible for conferring ACV resistance to HSV.

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Intracellular metabolism of acyclovir and deoxythymidine in cell culture system using HSV-1 and its acyclovir-resistant mutant strains. PK Bae, J.H. Kim, H.S. Kim, S-G. Paik[1], I.K. Chung[2] and C-K. Lee. Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology, Taejon 305-600, Korea; [1]Department of Biology, Chungnam National University, Taejon, Korea; and [2]Department of Biology, Yonsei University, Seoul, Korea.

To investigate the metabolism of acyclovir (ACV) and deoxythymidine (dThd) in cell culture system, we infected Vero and 143B cells with herpes simplex virus type 1 (HSV-1) strain F and AR1 – AR9, the laboratory derived TK-deficient ACV-resistant mutants of HSV-1 F. In HPLC assay, the level of ACV and dThd phosphorylation in AR1 infected cells was lower than HSV-1 strain F infected cells and similar to uninfected cells. The order of level of dThd phosphorylation in Vero and 143B cells was monophosphate > diphosphate > triphosphate, but in ACV was triphosphate ≥ diphosphate > monophosphate. We have measured viral DNA synthesis of the cell infected with virus and treated with ACV 0, 1, 2, 5, 10 $\mu g/m\ell$. Viral DNA synthesis which was surveyed by the radioactivity of incorporated [^{3}H]-Thd significantly increased from 6 h p.i. and reached its maximum 11 h p.i. We compared the intracellular uptake of dThd of cells infected with HSV-1 strain F or the ACV-resistant mutants. We examined the effect of ACV on intracellular uptake of [^{3}H]-Thd and its incorporation during the viral DNA synthesis. ACV increased the intracellular uptake of [^{3}H]-Thd in F- infected Vero cells, but decreased the incorporation of [^{3}H]-Thd.

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NEW QUANTITATIVE METHOD OF IN-LINE DESCRIPTION OF VIRUS-CELL INTERACTION AT EVERY STAGE

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Scope of the present work was to study the antiviral action of various preparations with the use of novel computer-assisted method of diffraction patterns fractal analysis (CA-DPFA). Fractal approach was chosen because it is stated experimentally that main systems and organs of human organism beginning from cellular level have fractal structure. Hep-2 cells cultivated for 24 and 48 hours were used as substrate for further infection with HSV-1 of US strain. E-aminocaproic acid (E-ACA) was used to modify the early stages by inhibition the stage of proteolysis. Aciclovir was used as the reference antiviral preparation. Fractal dimension D which has been changed during the viruses reproduction was determined using CA-DPFA dotated with the original software for image patterns processing. The antiviral action of the preparations under studies was examined independently with the use of standard technique of intra-nuclei virus specific inclusions direct counting through luminescent microscope. The main experimental result was that all examined diffraction patterns (DP) have shown a definitely fractal structure described fully by D values being strong specific for every type of specimen as well as for the prehistory of its preparation. We have registered also for the first time that almost all the information about virus-cell dynamic system behavior was concentrated in the central self-shadowed part of DP. The results of CA-DPFA quantitative analysis were found to be significantly consistent with those of direct counting of virus infected nuclei (Aciclovir caused decrease 80 % and E-ACA - 47.5 %) but CA-DPFA allows quantitative, objective and more accurate results.

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An Immunomodulating Peptide (CEL-1000) Elicits Protection Against HSV-1.

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Cel-1000 (DGQEEKAGVVSTGLIGGG) is an analogue of a peptide, which contains the CD4 binding domain of the beta chain of the human MHC Class II molecule in which the N-terminal asparagine has been replaced with aspartic acid and a triglycine added at the carboxyl terminus. Although covalent attachment of the native peptide to a T cell epitope and use as a L.E.A.P.S. conjugate vaccine promotes a Th2 type of response to the epitope, related studies suggest that CEL-1000 alone potentiates Th1 responses and protection in animals upon viral challenge. A/J mice (n=7-9) treated subcutaneously with a single dose of CEL-1000 (25 ug in 50 uL saline) emulsified in Seppic ISA-51 at 28, 21, 14, 7, 3, 1 or -1 days caused delay in the onset of symptoms and reduced both morbidity and mortality. Hundred percent protection was achieved when CEL-1000 was administered intramuscularly without adjuvant 14 days prior to challenge with HSV-1. CEL-1000 has been shown to induce protection against malaria and acts as adjuvant for an anti-tumor vaccine in relevant mouse models. CEL-1000 appears to activate innate and Th1 type immune responses to induce broad-spectrum protection against infection.

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In Vivo Anti-Herpes Simplex Virus Activity of Resveratrol, A Cyclin Dependent Kinase Gene Inhibitor

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Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a naturally-occurring phytoalexin found in grapes and other plants that has anti-HSV activity *in vitro* and inhibits the cell cycle at the S-G_2-M interphase (Antiviral Res. 43:145. 1999). We now demonstrate that resveratrol is effective *in vivo* and elucidate cell cycle targets of resveratrol required by HSV. The abraded epidermis of SKH1 mice were infected with HSV-1. After one hour, groups of animals (n = 8) were left untreated or treated with 12.5% or 25% resveratrol cream, cream only, 10% docosanol cream or 5% acyclovir ointment. 12.5% or 25% resveratrol applied every three hours five times per day for five days significantly ($p = 0.0001$) inhibited the development of HSV-induced skin lesions. Acyclovir was as effective ($p = 0.0001$) as resveratrol, but was applied every 2.5 hours six times a day for seven days according to the manufacturer's instructions. Even when treatment was not started until 12 hours after infection, 25% resveratrol remained effective. Animals that were treated with cream only or 10% docosanol developed severe lesions equivalent to untreated controls. Both 12.5% and 25% resveratrol inhibited lesion development by HSV-1 acyclovir-resistant virus. Resveratrol-treated animals showed no apparent dermal toxicity such as erythema, scaling, crusting, lichenification or excoriation. Cell cycle studies were initiated in order to determine the mechanism of HSV inhibition. Using cell cycle gene arrays and cDNA amplification of mRNA, relative signal strength of cell cycle genes in infected and uninfected cells in the presence and absence of resveratrol was measured. Resveratrol down-regulated a large number of cell cycle genes without significantly affecting housekeeping genes. Noteworthy was the inhibitory effect of resveratrol on cyclin-dependent kinases, particularly cdk-1 and -2, which are reported to be essential for HSV replication (J. Virol. 72:5626. 1998). These studies demonstrate that resveratrol is one of the first cyclin-dependent kinase gene inhibitors to be shown to be effective in vivo.

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In Vivo and In Vitro Antiherpetic Effects of Hydroxytolans

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Resveratrol (3,5,4'-trihydroxystilbene), a polyphenol, was shown to have antiherpetic activity (Antiviral Res. 43: 145, 1999). In order to search for resveratrol analogs with more effective antiherpetic activity, we designed and synthesized several polyphenol analogs and tested them for activity against herpes simplex virus type-1 (HSV-1). Polyphenol cytotoxicity was measured in Vero cells using a MTT assay. Studies on inhibition of viral replication were performed in Vero cells using a plaque assay. Three of the polyphenol analogs tested, hydroxytolans, were found to have anti-HSV activity. One of these three active hydroxytoians, a trihydroxytolan, was found to be more effective than resveratrol. This trihydroxytolan inhibited HSV-1 replication in a dose dependent and reversible manner. Reduction in HSV-1 yield by the trihyroxytolan was not through direct viral inactivation or by inhibition of virus attachment to the cell. The hydroxytolan seems to target an essential element in the immediate early phase of HSV replication. The inhibition of cell cycle genes vital to HSV-1 was measured using a cDNA gene array. Results showed that many of the cyclin dependent kinase (cdk) and all cyclin mRNA levels were depressed after hydroxytolan treatment of HSV- infected cells. In addition, there was an increase in the cdk inhibitor gadd45 and an increase in the cdk inhibitor p21 for hydroxytolan treated HSV-infected cells. In the presence of hydroxytolan, HSV-infected cells showed elevated mRNA levels of gadd45, p21, and reductions in both cdk1 and cdk2 known to be necessary for HSV replication. To test in vivo effectiveness of the trihydroxytolan, it was used as a topical treatment on HSV-1 infected skin of SKH-1 hairless mice. Results show that all control mice developed significant herpetic skin lesions, but mice treated with 50 mg/ml of the trihydroxytolan in DMSO did not. These studies demonstrate that the trihydroxytolan has significant anti-HSV activity in vitro and in vivo.

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Sulfonated halogenated porphyrins have potent virucidal activity against herpes simplex viruses 1 and 2 *in vitro* and *in vivo*.

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Topical virucides are a potential method of prevention of spread of viruses in general, but in particular of sexually transmitted viruses such as herpes simplex and human immunodeficiency viruses. In these studies, a variety of porphyrins were tested for virucidal activity against both HSV-1 and HSV-2. Very little activity was seen for the natural porphyrins (based on the protoporphyrin ring skeleton). Some sulfonated tetraphenyl and tetraarylporphyrins bearing carbon substituents show activity; sulfonated halotetraphenylporphyrins were in general much more active. Inhibition of infection was not due to a light-induced photodynamic effect in that all assays were carried out in the dark. The sulfonated tetra(2-F)TPP (TPP2F,S) was studied in the most detail. Inhibition of infection was evaluated in a mouse model using the compound in a 2% methylcellulose gel. TPP2F,S at 1 mg/ml in 2% methylcellulose was also able to inactivate approximately 99% of the inoculating virus. This family of molecules then shows promise as topical virucides capable of preventing transmission of genital herpes infections.

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Evaluation of Formulated Dendrimer SPL7013 as a Microbicide

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We have previously reported that dendrimers, highly branched macromolecules synthesized from a polyfunctional core, have in vitro and in vivo activity against HSV (Antimicrob Ag Chemother 44: 2471, 2000). We now report further on the lead microbicide candidate, SPL7013 (referred to previously as BRI 7013) formulated in a carbomer based gel vehicle. We have previously shown that this formulation containing 5% SPL7013 has activity in the mouse genital HSV-2 model when given 5 minutes prior to HSV-2 challenge. For the studies presented here we used both mouse and guinea pig models of genital HSV-2. In both models animals were pretreated intravaginally with the drug formulation, vehicle alone, or PBS at various times prior to intravaginal challenge with HSV-2. Protection was measured against disease (development of typical signs and symptoms) and infection (recovery of HSV-2 from the genital tract 24-48 hours after challenge). In the first study we extended the time from treatment to challenge to evaluate the duration of protection with a 1% SPL7013 formulation. Significant ($p<0.05$) protection against disease and infection respectively was seen at 5 (53%, 53%), 30 (60%, 53%) and 60 minutes (40%, 40%) in the treated group compared to placebo. We next extended the evaluations to the guinea pig model that more closely resembles the human disease. We compared the effects of treatment with 1-5% formulations in this model. Treatment at all five concentrations reduced infection and disease but there was an overall concentration effect. Protection was maximal at the 5% concentration providing 80% protection from disease and 73% from infection. When this experiment was repeated with the 3 and 5% gels an 89 and 94% protection was seen from disease and 83 and 90% protection was seen from infection. Dendrimer SPL7013 remains a leading microbicide candidate. Further studies to explore its efficacy against SIV and chlamydia in non human primate models are underway.

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Abreva‰ (10% n-docosanol cream) vs 5% Acyclovir (ACV) in 80% Dimethyl Sulfoxide (DMSO) in the Treatment of Experimental Dorsal Cutaneous Herpes Simplex Virus 1 (HSV-1) Infection in the Hairless Guinea Pig. M. McKeough* & S. Spruance*. *University of Utah , Salt Lake City, UT USA.

Abreva(TM) (10% n-docosanol cream) is approved by the FDA as an over-the-counter topical treatment for herpes simplex labialis (J Amer Acad Derm 2001;45:222). N-docosanol, a 22-carbon straight-chain saturated alcohol, has anti-inflammatory properties (J Invest Derm 1987;89:380; Cont Derm 2000;43:79) but no activity in the Hartley guinea pig model of cutaneous HSV-1 infection (Arch Derm 2001;137:1153J). Because of claims by the manufacturer that use of the hairless guinea pig (41st ICAAC, Abstract #H1896) is a superior model to the haired, Hartley animal, we repeated our Abreva studies in hairless animals. Briefly, four areas on the backs of female hairless guinea pigs were infected by application of a suspension of HSV-1 to the skin followed by multiple activations of a vaccination device (Day 0). Abreva was compared to untreated control (Untx) sites. 5% ACV in 80% DMSO compared to 80% DMSO was run concurrently as a control. Treatments were evaluated for efficacy on Day 4 by measuring lesion number, total lesion area and virus titer. Both Abreva and ACV were applied 4x/day for three days.

Measure	Abreva	Untx	ACV	DMSO
No. of lesions	43	38	31*	39
Total Lesion Area (mm2)	151	139	73*	125
Lesion Virus titer (log10pfu/ml)	4.23	3.93	3.34*	4.18

* = p<.05 vs control infection site; n = 12 infection sites; median values.

Abreva showed no efficacy in any of the parameters measured, confirming previous results in our model using haired guinea pigs (Arch Derm 2001;137:1153) and eliminating depilation as a confounding factor. ACV in DMSO compared to DMSO alone significantly reduced lesion number, total lesion area and lesion virus titer by 20% (p = .004), 42% (p = .002) and 85% (p = .002), respectively. When the results for Abreva and ACV were compared to each other by the Mann-Whitney rank sum test, ACV was statistically significantly better than Abreva in all three measures. A growing body of evidence indicates that the small clinical activity of Abreva may be due to its anti-inflammatory properties and not an antiviral process (Amer Acad Derm 2002;47:457).

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Antiviral activity of *cyclo*Saligenyl prodrugs of BVDU against herpesviruses (HSV, VZV, HCMV, EBV)

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A series of 35 lipophilic 5-bromovinyl-2'-deoxyuridine monophosphate (BVDUMP) derivatives are presented as potential prodrugs of the antiviral agent BVDU. To this compound the 5'-*cyclo*Sal-masking group technique has been applied in order to achieve the delivery of the monophosphate of BVDU inside the target cells and to increase membrane penetration. The new compounds have been tested for their antiviral activity against herpes simplex virus type 1 (HSV-1), thymidine kinase-deficient (TK^-) HSV-1, HSV-2, varicella-zoster virus (VZV), human cytomegalovirus (HCMV) and Epstein-Barr Virus (EBV). Using the XTT-based tetrazolium reduction assay EZ4U the derivatives were examined for their antiviral and cytotoxic activities in HSV-1, HSV-1-TK^- as well as HSV-2 infected Vero cells. The plaque reduction assay was applied for proving the anti-VZV and anti-HCMV activity in primary human embryonal lung fibroblasts. The anti-EBV activity was assessed by means of an EBV DNA hybridization assay using a digoxigenine-labelled probe specific for the Bam HI-W-fragment of the EBV genome in P3HR-1-cells. The results indicate that all compounds tested proved to be potent and selective inhibitors of HSV-1 and VZV replication at concentrations that were not toxic to the host cells. No antiviral effect could be demonstrated against TK^-HSV-1, HSV-2 and HCMV. Surprisingly, a few *cyclo*Sal-BVDUMP triesters yielded promising anti-EBV activity while the parent compound BVDU was entirely inactive.

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Interaction of the Bicyclic Nucleoside Analogues (BCNAs) with Human Varicella-Zoster Virus (VZV) and Simian Varicella Virus (SVV) Thymidine Kinase and VZV DNA Polymerase.

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Bicyclic nucleoside analogues (BCNAs) are potent and selective inhibitors of varicella-zoster virus (VZV) replication in HEL cell cultures. They are inactive against the closely related herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), or any other DNA or RNA virus. The VZV-encoded thymidine kinase (TK) is essential for the anti-VZV activity of the BCNAs. We found that, in contrast to the HSV-1 TK, purified VZV TK converts BCNAs to BCNA 5'-monophosphate and BCNA 5'-diphosphate. Simian varicella virus (SVV, *Cercopithecus herpes virus 7*) shares many properties with human VZV. It causes a natural varicella-like disease in non-human primates. Due to the close similarities between VZV and SVV, SVV infection in nonhuman primates can be used as a representative VZV animal model. We considered the possibility of this monkey model to examine the antiviral efficacy of BCNAs. However, the BCNAs were not inhibitory to SVV replication in BSC-1 cells. Interaction studies of the BCNAs with SVV TK is currently under investigation to reveal the molecular basis of the lack of susceptibility of this virus towards the BCNAs. As BCNAs obligatorily need to be phosphorylated by VZV TK to display anti-VZV activity, VZV DNA polymerase is the most plausible final target. Therefore the BCNA-5'-triphosphate derivative was synthesized and evaluated for its inhibitory action against purified VZV DNA polymerase. The detailed kinetics of BCNA-5'-triphosphate with VZV, HSV-1 and cellular DNA polymerases are under investigation.

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Potent antiviral activity of red microalgal polysaccharide against *Varicella zoster virus*

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The effect of the sulfated polysaccharide obtained from red microalgae *porphyridium* sp. (*Psp*) on the development and progress of infection caused by *Varicella zoster* virus (VZV) was examined *in vitro*. The experiments showed that concentrations of ≥100μg/ml of the polysaccharide completely prevented viral infection and development of the cytopathic effect. There were no cytotoxic effects in the control noninfected cells, even at concentrations of polysaccharide as high as 1,000 μg/ml. When the polysaccharide was added to cultures that had already been infected with VZV, development and progress of the cytopathic effect was inhibited. The antiviral activity of the red microalgal polysaccharide against VZV was superior to that exhibited by other sulfated polysaccharides, such as dextran sulfate and carrageenan (an algal polysaccharide). Our studies showed a strong interaction between the polysaccharide and VZV, and a weak interaction between the polysaccharide and the cell membrane. The sulfated polysaccharide/virus interaction might be responsible for preventing adsorption of the virus to the host cells. It was also evident that the polysaccharide inhibited the production of virus particles in cell cultures infected before its application. Results from our FTIR spectroscopy showed a significant accumulation of carbohydrates in infected cells treated with the polysaccharide, which possibly plays a role in inhibiting the replication of the virus. These results suggest that the sulfated algal polysaccharide might affect more than just one step in the replication cycle of herpes viruses. Therefore, it appears to be a very attractive agent for antiviral therapy since resistant mutant viruses may be less likely to develop in response to drugs that have a pleiotropic mode of action in the life cycle of the virus.

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Penetration of Highly Active Anti-VZV Nucleoside Analogues Into and Through Full-Thickness Skin

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Varicella Zoster Virus (VZV) causes chickenpox (primary infection) and shingles (secondary infection). Reactivation can lead to severe post-herpetic neuralgia (PHN), which may last for years and is frequently poorly responsive to treatment. The best antiviral treatments available have limited effect on shingles or PHN, however a class of nucleoside analogues has been discovered in our laboratories that show potencies against VZV in cell culture that are up to 30,000 times higher than aciclovir, and are highly selective. They are highly lipophilic, which lends them ideally to use in topical dosage forms. Three compounds from the series were formulated into creams, and their penetration into and through full-thickness pig ear skin (a good model of human skin) was evaluated over 48 hours. The aim was to target the dermatomal site of viral replication during secondary infection - the basal epidermis. Cf1698 penetrated through the skin into the receptor phase, predicting an ability to enter the systemic circulation, while Cf1743 and Cf1712 did not. All three compounds however reached the target site in the epidermis in concentrations far greater than their EC_{50} values. Laser–scanning confocal microscopy was also used to visualise the compounds in skin after 24-hour experiments, and again they were seen to have penetrated deep into the epidermis. These compounds are promising agents for the first line topical treatment of VZV infections. By attacking the virus in this way at the first sign of reactivation, it is proposed that the extent of damage caused by the virus would be significantly lowered, thereby limiting the extent and severity of PHN.

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Cell death induced by (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) in varicella zoster virus thymidine kinase expressing cells: activation of c-Jun/AP-1 and FasL/caspase-8

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The molecular mode of cell killing by the antiviral drug (E)-5-(2-bromovinyl-2'-deoxyuridine (BVDU) was studied in Chinese hamster ovary (CHO) cells stably transfected with the thymidine kinase gene (*tk*) of varicella zoster virus (CHO-VZV*tk*). Colony formation of the cells was reduced to <1% at a concentration of ~1 μM BVDU while non-transfected cells or cells transfected with the *tk* gene of herpes simplex virus type 1 (CHO-HSV*tk*) were 1000-fold more resistant to the drug. BVDU was incorporated into the genomic DNA of both VZV*tk* and HSV*tk* expressing cells to nearly equal amounts, however, it inhibited thymidylate sythase (TS) in CHO-VZV*tk* but not in CHO-HSV*tk* and control cells. Since exogenous thymidine protected from BVDU-induced cell killing, cells obviously die because of thymidine depletion. At highly cytotoxic BVDU concentrations (50 μM) VZV*tk* cells were blocked to some extent in S- and G2/M-phase (24-48h) and finally underwent apoptosis and necrosis (24-72h of drug exposure). The apoptotic pathway was studied in detail revealing that BVDU triggers induction of c-Jun and activation of the transcription factor AP-1 resulting in increased expression of Fas ligand (FasL) and enhanced activation of caspase-8/-3. Consequently, Bid and PARP were cleaved. Expression of Bax increased while Bcl-2/Bcl-x_L remained unchanged. Transfection of dominant-negative FADD and inhibition of caspase-8 by zIETD-fmk strongly abrogated BVDU-induced apoptosis indicating the Fas/CD95/Apo-1 system to be crucially involved. Thus BVDU-triggered apoptosis differs significantly from that induced by ganciclovir which activates in the same cellular background the mitochondrial damage pathway.

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Varicella zoster virus infection induces cyclin dependent kinase 2/cyclin E expression and replication is blocked by roscovitine, a cdk2 inhibitor.

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Roscovitine inhibits cdk1, cdk2, and cdk5, and also blocks replication of VZV and other viruses. This antiviral mechanism was not understood, so we investigated the effects of rosco on VZV gene expression and the effects of VZV infection on cyclin dependent kinases, targets of rosco inhibition. Here, we demonstrate a link between VZV infection and cdk2 expression in non-dividing HFF cells that is the first step in elucidating the mechanism of rosco's antiviral activity. Initial studies using MeWo cells showed that the IC_{50} of rosco for VZV replication was approximately 12μM by plaque reduction assay. Using Real Time PCR, rosco was shown to inhibit VZV genomic DNA synthesis with an IC_{50} of 14μM. Rosco also caused downregulation of VZV mRNA, suggesting that rosco prevented viral DNA replication by shutting down viral gene expression. In HFF cells, confocal microscopy showed that rosco treatment restricted the expression of immediate early proteins 4, 62, and 63, major viral transactivators. In untreated cells, VZV infection resulted in a dramatic induction of cdk2 and cyclin E. Cdk2/cyclinE regulates the transition from quiescence to cell division and is normally absent in confluent HFF's. These results are consistent with the hypothesis that rosco inhibits a cdk, possibly cdk2, that is required for initiation of VZV gene expression at very early times after infection.

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Antiviral Structure-Activity Relationships of the First and Second Generation Methylenecyclopropane Nucleoside Analogues.

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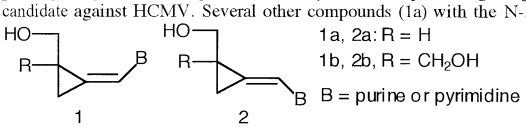
Recently, we have developed two series of methylenecyclopropane analogues of nucleosides 1a and 1b effective against CMV and EBV. The first generation series 1a has progressed to preclinical development stage. The 2-amino-6-cyclopropylaminopurine derivative, a potential prodrug of guanine analogue, is currently the most promising drug candidate against HCMV. Several other compounds (1a) with the N-,



O- and S-alkyl purine-6 substituents (total of 14 new analogues were investigated) were strongly effective against HCMV in culture and, in some cases, also against EBV, VZV, HSV-1, HSV-2 and HBV. For example, N^6-allyl, O^6-pentyl and S^6-propyl analogues had EC_{50}/CC_{50} (μM) against HCMV(Towne and AD169)/HFF cytotoxicity 1.8-2.8/>100->368, 0.21-1.4/>100-178 and 0.22-3.0/>100->343. The range of antiviral potency of the second generation series 1b (8 analogues tested) was narrower than that of 1a. The guanine analogue was the most effective against HCMV in culture but, surprisingly, the 2-amino-6-cyclopropylamino compound was inactive. In contrast to the *E*-series 2a where only adenine and cytosine analogues were effective against EBV, several compounds of the *E*-series 2b (8 analogues tested) were potent inhibitors of EBV or VZV. Guanine analogue was the most potent inhibitor of VZV/HFF (EC_{50}/CC_{50} 0.65/>368 μM) and 2-amino-6-cyclopropylamino analogue inhibited EBV/Daudi with 0.30/>165 μM. Supported by NIH grants RO1-CA32779, RO1-AI44358, PO1-AI46390 and contract NO1-AI85347.

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In Vitro Activity of Methylenecyclopropane Analogs of Nucleosides against Herpesvirus Replication.

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We have reported previously that purine methylenecyclopropane analogs have demonstrated good activity against the herpesviruses. In an attempt to improve the activity of these compounds, the 2-amino-6-cyclopropylaminopurine analog was selected for the study of structure-activity relationships by modifying the purine 6 substituent for the XCC series of compounds. The ZSM series is the second generation of methylcyclopropane analogs, the 2, 2-bis-hydroxymethyl derivatives. It is based on an alkylation-elimination approach discussed previously for 2-(hydroxymethyl) methylenecyclopropanes. Nine compounds from the XCC series and five compounds from the ZSM series were tested against human and murine cytomegalovirus (HCMV, MCMV), herpes simplex virus types 1 and 2 (HSV-1, HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6) and human herpesvirus 8 (HHV-8). All except two compounds were active against HCMV and MCMV (0.5–14μM and <0.1–10μM respectively). Five compounds from the XCC series and two from the ZSM series were active against VZV (0.6–12μM). One compound from the XCC series and none from the ZSM series were active against HSV-1 and EBV (6.3μM and 0.6μM respectively). Six compounds from the XCC series and one from the ZSM series were active against HHV-6A (1–10μM). Five compounds from the XCC series and two from the ZSM series were active against HHV-6B (1–5μM). Seven compounds tested from the XCC series were active against HHV-8 (1–6μM). Toxicity was evaluated in adherent and non-adherent cell lines and minimal toxicity was observed. These results indicate that the methylenecyclopropane analogs are highly active against the β herpesviruses, HCMV, MCMV, HHV-6A, and HHV-6B but were less active against the α herpesviruses, HSV-1, HSV-2, and the γ herpesviruses, EBV and HHV8.

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Synthesis and Biological Evaluation of Bicyclic Furano Pyrimidine Nucleosides as Inhibitors of Human Cytomegalovirus

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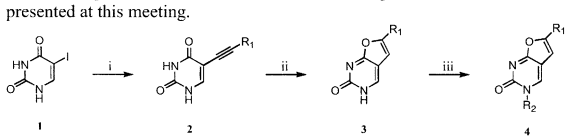
Ganciclovir, cidofovir and foscarnet, the three main FDA approved drugs used presently in the treatment of human cytomegalovirus (HCMV) exert their influence as DNA polymerase inhibitors. However, problems such low oral bioavailability and toxicity highlight the urgent need for alternative treatments. Here, we present a series of novel non-cytotoxic bicyclic furo[2,3-*d*]pyrimidines which appear to display a comparative potency *in vitro* against HCMV. Compounds **4** were prepared from their parent furano pyrimidine bases **3** (**Scheme 1**). Bases **3** were obtained *via* the standard protocol of Cu/Pd coupling of the various alkynes to 5-iodouracil **1** and subsequent cyclisation of intermediate **2** with Cu catalyst and base formed the desired bicycles in a one-pot synthesis. The corresponding bases **3** were then reacted with a suite of alkylating agents to form the desired N-alkylated compounds **4**. The antiviral evaluation of prepared compounds **4** has found that these compounds are as active as ganciclovir, having an IC_{50} at 1-2 μM concentrations. They are some 30 times more potent than foscarnet, and 10 times less potent than cidofovir. Further biological evaluation will be presented at this meeting.



Scheme 1 (i) 1-alkyne, DIPEA, $Pd(PPh_3)_4$, CuI, RT, 16 h; (ii) CuI, Et_3N, 120 °C, 6 h; (iii) R_2X, DMF, 80 °C, 5-10 h.

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Synthesis and Biological Evaluation of a New Series of 5-Alkylaryl Nucleoside Analogues Showing Anti-HCMV Activity.

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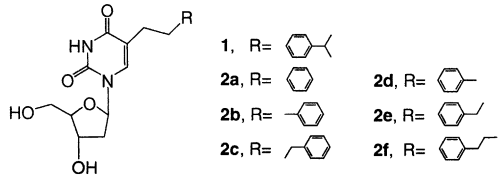
Bicyclic furano pyrimidines have been discovered in our laboratories to be potent and selective anti-VZV nucleoside analogues. Following SAR studies aimed to elucidate optimal requirements for biological activity, we observed that the bicyclic base undergoes opening of the furo ring in the presence of H_2 and Pd/C leading to the non fluorescent saturated 5-alkyl compound. Biological data related to a series of 5-alkyl-2'-deoxyuridines showed activity as inhibitors of HSV-1 TK, but they have been found to be inactive against HSV-1. Moreover, derivative **1**, bearing a phenyl ring in the side chain, was tested against HCMV AD-169 and showed unexpected antiviral activity with EC_{50}=5μM and CC_{50}=182μM. In order to widen the studies for this class of compounds, the synthesis of a new series of derivatives has been initiated. Targets compounds **2a-2f** will provide information about the effects of the length of the side chain bearing a phenyl ring moving throughout the chain. The synthetic pathway involves a Pd(0)-catalysed coupling of 5-iodo-2'-deoxyuridine with appropriate terminal alkynes to give the corresponding 5-alkynyl nucleosides which are subsequently hydrogenated in the presence of H_2 and Pd/C.
The synthesised compounds are now undergoing biological screening and the data will be presented at the meeting.



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Alkoxyalkyl Esters of Adefovir: Antiviral Activity Against Cytomegalovirus and HIV-1, *In Vitro*

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Adefovir (9-[2-phosphonylmethoxyethyl]adenine) (ADV) has been found to be an effective antiviral against a wide range of viruses such as human immunodeficiency virus (HIV-1), other retroviruses, herpesviruses and hepatitis B virus. Previous work in our laboratory has shown that long chain alkoxyalkyl esters of nucleotide phosphonates not only have enhanced antiviral activity but are also orally bioavailable. For this study, two lipid analogs of ADV were synthesized, 1-O-hexadecyloxypropyl-ADV (HDP-ADV) and 1-O-octadecyloxyethyl-ADV (ODE-ADV), and tested against human cytomegalovirus (HCMV) infected MRC-5 cells. HDP-ADV and ODE-ADV exhibited a 700-2000 fold increase in activity over unmodified ADV. The EC_{50} values were also greatly enhanced with a 1000-1400 fold increase in a plaque assay using HIV-1 infected HT4-6C cells and a roughly 50,000 fold increase in potency in a p24 reduction assay in MT-2 cells. These results show that HDP-ADV and ODE-ADV are promising new antiviral candidates that warrant further evaluation.

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Synthesis of Alkoxyalkyl Esters of (R)- and (S)-HPMPA and Antiviral Activity Against Herpesviruses, *In Vitro*

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Long chain alkoxyalkyl esters of cidofovir (CDV) have been previously shown to exhibit enhanced antiviral activity and selectivity *in vitro* against human cytomegalovirus (HCMV) and against orthopoxviruses compared with underivatized CDV. The related nucleoside phosphonate, 9-(S)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA), has also been reported to have substantial activity against herpesviruses. To extend our study of nucleoside phosphonate ether lipids, we synthesized and evaluated 1-O-hexadecyloxypropyl-HPMPA (HDP-HPMPA), 1-O-oleyloxyethyl-HPMPA (OLE-HPMPA) and 1-O-oleyloxypropyl-HPMPA (OLP-HPMPA) for antiviral activity and selectivity against herpes simplex virus (HSV-1) and HCMV by DNA reduction assays (Diagnostic Hybrids) in MRC-5 human lung fibroblasts. Against HSV-1, the ether lipid analogs were highly active with EC_{90} values of 4×10^{-4}, 9×10^{-4} and 25×10^{-4} μM for HDP-HPMPA, OLE-HPMPA and OLP-HPMPA, respectively. The analogs were also highly active against HCMV. Compared to the reported results for (S)-HPMPA, the compounds appear to be $>10^3$ fold more potent. The corresponding esters of the (R) enantiomers were significantly less active. The (S)-HPMPA analogs appear to be good candidates for further evaluation as possible oral therapies for herpesvirus diseases.

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Anticonvulsive drug valproic acid increases replication of HCMV in human foreskin fibroblastes and retinal pigment epithelial cells in therapeutic concentrations

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Valproic acid (VPA), the mostly prescribed antiepileptic drug worldwide, is a potent histone deacetylase (HDAC) inhibitor. It was already shown to increase replication of human cytomegalovirus (HCMV) in human fibroblasts. This report shows for the first time that HCMV replication can be increased by VPA at concentrations between 0.5 and 1 mM. Such concentrations are in the range of plasma levels of VPA treated patients. HCMV replication was observed in human foreskin fibroblasts (HFF) and retinal pigment epithelial (RPE). The replication induction was time- and concentration-dependent after preincubation with VPA and it was preceeded by increase of HCMV immediate early antigen (IE) expression. No effect was found when VPA was added after virus infection. Different VPA derivatives increased to a different extent HCMV IE expression. The stimulatory effects of the derivatives correlated with their HDAC inhibition. The data suggest that VPA stimulates HCMV replication the mechanism of which might involve HDAC inhibition. The HCMV stimulation might be of clinical relevance as it was achieved with therapeutical VPA concentrations.

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In Vivo Activity of 1-O-Hexadecyloxypropyl-cidofovir (HDP-CDV) against Murine and Human Cytomegalovirus Infections.

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Human Cytomegalovirus (HCMV) infections in normal and immunocompromised hosts can cause severe clinical manifestations. Although ganciclovir and cidofovir (CDV) are effective therapies for HCMV, there is a need for compounds that are active orally. To improve the oral bioavailibity of CDV, a series of alkoxyalkyl esters were synthesized and one of the most active compounds, HDP-CDV, was evaluated using murine CMV (MCMV) and HCMV infections in mice. In Balb/c mice inoculated with MCMV, oral treatment once daily with HDP-CDV for 5 days starting either 24 or 48 hours after infection provided significant protection from mortality. The activity of HDP-CDV was also examined in two models of HCMV infection in which SCID mice were implanted with human fetal tissue which was subsequently infected with HCMV. In one model, retinal tissue was implanted into the anterior chamber of the SCID mouse eye and infected 6 weeks later with 4400 pfu/eye. Treatment orally with vehicle, 10, 5, or 2.5 mg HDP-CDV/kg or intraperitoneally with 20 mg CDV/kg was begun 24 hours after infection and continued once daily for 28 days. The results indicated that by 28 days after infection, virus titers in the vehicle treated group were 168 ± 127 pfu/ml compared to 17 ± 14 pfu/ml in the CDV-treated group. In groups treated with HDP-CDV, a dose response was observed with titers ranging from 11 ± 11 pfu/ml in the high dose group to 97 ± 88 pfu/ml in the low dose group. In the second model, thymus and liver tissue was implanted under the kidney capsule and infected 16 weeks later with 6600 pfu/implant. Starting 24 hr after infection, animals were treated as above with the exception of CDV which was administered at 10 mg/kg. The results indicated that by 28 days after infection, HCMV titers in the vehicle-treated group were 5.39 ± 5.49 $\log_{10}$ pfu/g. In comparison, titers from the CDV-treated group were 4.55 ± 5.05 $\log_{10}$ pfu/g and no virus was detected in any of the HDP-CDV-treated groups. These studies suggest that HDP-CDV has significant activity against MCMV and HCMV in vivo and that this compound is an excellent candidate for further evaluation.

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BAY 38-4766: An Effective Antiviral Agent Against Guinea Pig Cytomegalovirus (GPCMV). M Schleiss, S Hallenberger, K Henninger, FJ Bravo, G Stroup, and DI Bernstein. Children's Hospital Med. Center, Cincinnati, OH and Bayer Corporation, Cambridge, MA.

Better treatment options for cytomegalovirus (CMV) infections are urgently needed. The strict species-specificity of CMVs, however, precludes study of human CMV in animals, and requires study of animal CMV models for preclinical antiviral testing. Guinea pig CMV (GPCMV) is a useful model, given the similarities in pathogenesis with HCMV, including its ability to cross the placenta and cause infection *in utero*. We evaluated a novel selective nonnucleoside inhibitor of CMV replication, BAY 38-4766, for activity against GPCMV *in vitro* and *in vivo*. Plaque reduction established an IC_{50} for BAY 38-4766 of 0.5 μM, similar to the activity observed against HCMV. Yield reduction assays determined an ED_{90} and ED_{99} of 0.4 μM and 0.6 μM, respectively. Guinea pigs tolerated oral admininstration of 50 mg/kg/day of BAY 38-4766 without evidence of biochemical or hematologic toxicity. Plasma concentrations of BAY 38-4766 were high following oral dosing, with a mean peak level 1 hour post-dose of 26.7 μg/ml (n=6; range, 17.8-35.4). BAY 38-4766 crossed the placenta in pregnant guinea pigs, with fetal plasma concentrations amounting to 20-30% of maternal levels. BAY 38-4766 was evaluated for protection against GPCMV infection in strain 2 guinea pigs immunocompromised with cyclophosphamide (CP). Animals were treated with CP, 200 mg/kg, and following inoculation with GPCMV (4×10^5 pfu subcutaneously) were treated with either BAY 38-4766, 25 mg/kg BID (n=6), or vehicle (n=7), for 7 days. On day 10 post-inoculation, blood for culture and quantitative competitive PCR (qcPCR) assay was obtained. Viremia was reduced in BAY 38-4766 treated animals, with a mean titer of 10^2 $TCID_{50}$/ml in treated animals, compared to $10^{3.14}$ $TCID_{50}$/ml in controls. QCPCR identified higher viral load in the placebo group; median viral load was 5.42 $\log_{10}$ genomes/ml, compared to 4.89 $\log_{10}$ genomes/ml in treated animals ($p<0.04$, Mann-Whitney test). Based upon its favorable safety and pharmacokinetic profiles, and its transplacental passage, BAY 38-4766 warrants further investigation in the GPCMV model. Funding: NIH AI15439.

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Effective Treatment of Congenital Cytomegalovirus (CMV) by Cyclic HPMPC using the Guinea Pig Model of Congenital CMV

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CMV is the most common congenital infection in the developed world. We have previously shown that cyclic HPMPC (cHPMPC) was safe and effective in an immunocompromised model of guinea pig CMV (gpCMV) infection when given in a single dose (Antiviral Res. 47:103, 2000). We now evaluate whether protection occurs in the gpCMV model of congenital infection in which virus crosses the placenta of guinea pigs producing 50-100% mortality in the pups. Pregnant Hartley guinea pigs were infected with virulent gpCMV (~ 5 log pfu) during the second/third trimester. Guinea pigs received either cHPMPC (35 mg/kg, IP, once) (N=12) or placebo (N=12). Animals were followed through delivery and the number of liveborn and stillborn pups evaluated. Tissues from liveborn and stillborn pups harvested within seven days after birth were evaluated by PCR for gpCMV infection. Tissues from liveborn pups were also cultured for gpCMV. All of the dams survived infection. Of the 12 litters born to placebo treated animals, 11 of 39 pups were liveborn (28.2%), while in the 12 litters of treated animals, 36 of 43 pups were liveborn (83.7%), ($P<0.001$). Virus was recovered from 2/9 liveborn placebo animals and 7/27 treated animals that were cultured. PCR analysis is not yet complete. Treatment also extended the duration of pregnancy from 13.6 to 24.4 days after gpCMV inoculation. ($P<0.01$). Antiviral therapy decreased the mortality of congenital CMV infection but did not prevent infection of the pups. This is the first evidence that antiviral therapy can modify the outcome of congenital CMV infection.

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Characteristics of Epstein-Barr Virus encoded protein kinase

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Among the different human herpesvirus protein kinases (PK's), the BGLF4 gene, which encodes Epstein-Barr Virus PK, is the least studied. It was first identified as a Ser/Thr protein kinase-related gene using amino acid sequence alignment of regions conserved within the catalytic domains of protein kinases. The BGLF4 gene is the only potential protein kinase identified in the EBV genome. Recently, we and others showed protein kinase activity associated with BGLF4-encoded protein, and we have shown that this kinase is involved in hyperphosphorylation of the EBV DNA polymerase processivity factor, EA-D; however, the characterization of EBV PK remains incomplete. In this study we fill several gaps in knowledge about EBV PK: i) EBV PK is detected by immunoblotting as a single band at ~49 kD (predicted M(r) is 51 kD); ii) the protein is expressed as early as 4 hours after viral reactivation in Akata cells and peaks between 12 and 24 hours and is classed as an early protein; iii) the protein is localized in the nucleus; iv) its biochemical properties are somewhat distinct from those reported for its close homologs, including the fact that EBV PK activity itself was not inhibited by maribavir and indolocarbozoles shown to inhibit its close homolog HCMV UL97. Thus, these data suggest that even closely related gene products of different herpesviruses can have distinct characteristics, and may implicate different roles for these homologs in the virus life cycle. The study of this diversity will help to identify new targets for antiviral therapy.

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Studying of antiEpstein-Barr virus activity of 6-azacytidine and its acyclic derivative

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Modified nucleosides have proved to be the useful substances for antiviral and anticancer drug design. 6-Azacytidine (2-β-D-ribofuranosyl-5-amino-1,2,4-triazin-3(2H)-on; 6-AC) is an original structural cytidine analogue. The objective of the present investigation was to study the activity of 6AC against Epstein-Barr virus - lymphotropic and oncogenic virus from Herpesviridae family. The antiEBV-activity of 6-AC acyclic derivative - 2'3'-"seco"-5 methyl-6 Azacytidine (seco 6-AC) was also studied. As a model of EBV-infection in vitro we used the line of lymphoblastoid B-cells Raji, which infected by EBV. An inhibition of reproduction of EBV in cell culture by 6-AC and seco 6-AC was determined by reduction of a number of genome – equivalents of EBV DNA on a cell, which were revealed by quantitative PCR with use of primers and reagents "Amply-Senc-100 R" (Russia). First stage of investigation of 6-AC and seco- 6AC was the analysis of their cytotoxicity for cell line Raji. We have studied 6-AC and seco 6-AC in concentrations of 250, 125, 64, 32, 16, 4, 1, 0,5 and 0,1 μg/ml. The concentrations which inhibited the quantity of alive cells on 50% (ID_{50}) were equal to 125 μg/ml for both substances. The minimal inhibiting concentration (MIC) of 6-AC was equal to 0,5 μg/ml, because the amount of genome - equivalents of DNA EBV on a cell were reduced with 6,0 up to 3,1. MIC for seco 6-AC was equal to 1 μg/ml (the amount of genome - equivalents were reduced with 7,2 up to 2,9). Hence, the index of selectivity (IS) was equal to 250 and 125 for 6-AC and seco 6-AC accordingly. As it has been shown by us earlier (Dyachenko et al, 1999; Alexeeva et al, 2001; Nosach et al, 2002), IS of 6-AC was 62-125 toward to adenoviruses types 1,2 and 250 towards to adenoviruses type 5 and herpes simplex virus type 1. It was investigated the influence of 6-AC and seco 6-AC on CD 95 – mediated apoptosis in Raji cells infected by EBV. The presented investigation study was partially supported by INTAS Grant Program (INTAS Grant N 011-2382)

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Cytogenetic genotoxicity of new cycloSaligenyl prodrugs of ganciclovir and aciclovir.

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The aim of the present study was to investigate the properties of two cycloSaligenyl (cycloSal) monophosphates (MP) of the acyclic purine nucleoside analogues aciclovir (ACV) and ganciclovir (GCV) for their cytogenetic genotoxicity in comparison to the parent compounds in CHO cells (CHO-neo) and in herpesvirus thymidine kinase gene-transfected CHO cells (CHO-HSVtk$^+$).
These potent lipophilic, membrane-soluble pronucleotides selectively release ACV-MP and GCV-MP by a tandem reaction (Meier et al., 1998). In this way, they form a so-called "thymidine kinase bypass".
The compounds were applied for the duration of one cell cycle (14h), and the effects for clastogenic and sister chromatid exchange (SCE)-inducing activities were measured 28 h later.
GCV and the cycloSal-GCV-MP were very potent genotoxins in CHO-neo cells. The genotoxic activity (SCE induction and structural aberrations) of the GCV pronucleotide in the CHO-neo system was 10 fold higher that of GCV.
ACV induced borderline effects in both cell systems. Nevertheless, the genotoxic activity of cycloSal-ACVMP is higher than that of ACV in CHO-neo cells.
In conclusion, the two cycloSaligenyl (cycloSal) monophosphates (MP) of acyclic purine nucleoside analogues aciclovir (ACV) and ganciclovir (GCV) showed a higher genotoxic activity in non-transfected CHO system than the parent compounds.
The biological activities of these pronucleotides were similar to that of ACV and GCV in herpesvirus thymidine kinase gene-transfected CHO cells. The results confirm the high biological activity of cycloSal derivatives in thymidine kinase deficient viruses.

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Inhibitory Effect of Alkoxyalkyl Esters of Acyclic Nucleoside Phosphonates Against Orthopoxvirus Replication.

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In an attempt to obtain better oral activity, while retaining or increasing antiviral potency against orthopoxvirus replication, a series of analogs were synthesized by esterification of the parent acyclic nucleoside phosphonates cidofovir (CDV), cyclic cidofovir (cCDV), 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [(S)-HPMPA], and 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG). Using a plaque reduction assay, these analogs were evaluated against Vaccinia Virus (VV) and Cowpox Virus (CV) in tissue culture cells. Previously tested analogs 3-hexadecyloxy-1-propanol (HDP-CDV; HDP-cCDV) and 3-octadecyloxy-1-ethanol (ODE-CDV;ODE-cCDV) were found to be several-fold more active in vitro than the parent compounds. Results from newly synthesized analogs of CDV and cCDV, including 1-O-oleylethanediol (OLE-CDV;OLE-cCDV) and 1-O-oleylpropanediol (OLP-CDV;OLP-cCDV), indicated these compounds were among the most active tested in this series, with EC_{50} values ranging from 0.06 – 0.5 μM against both viruses. Comparatively, these analogs were 65 – 650 fold more active than the parent compounds. Other ester analogs of CDV and cCDV, including ODP, OPD, ODGB and HD, were found to be equally or less active than the OLE-OLP compounds. The (S)-HPMPA analogs [HDP-(S)-HPMPA; ODE-(S)-HPMPA] had average EC_{50} values of 0.01 μM for both VV and CV. These values were over 100 fold greater than the numbers for (S)-HPMPA. The PMEG analogs (HDP-PMEG; ODE-PMEG) exhibited similar results, with average EC_{50} values of 0.1 μM, resulting in >100 fold increases over the parent compound. Many of these new analogs have enhanced activity against orthopoxvirus replication *in vitro*, and warrant further investigation for determining their oral absorption and efficacy in animal models.

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Effects of 9-[2-(Phosphonomethoxy)ethyl]guanine (PMEG), Hexadecyloxypropyl-PMEG and Octadecyloxyethyl-PMEG on Replication of HIV-1, Herpesviruses and Poxviruses, *In Vitro*

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Drug delivery to the posterior segment of the eye by intravitreal injection or intravitreal implant has been used to treat acute and chronic vitreoretinal diseases. Nucleoside analogs (e. g. ganciclovir) are active against viral infections of the retina, but rapid clearance from the vitreous cavity after intravitreal injection limits their effectiveness. We previously reported that an alkoxyalkyl ester of ganciclovir monophosphate (HDP-P-GCV) releases GCV into the vitreous and provides a 20-week antiviral effect in an HSV rabbit retinitis model following a single intravitreal injection. To extend this method to delivery of acyclic nucleoside phosphonates, we synthesized alkoxyalkyl derivatives of 9-[2-(phosphonomethoxy)-ethyl]guanine (PMEG), a guanine nucleotide analog with broad spectrum antiviral and antiproliferative activity. 2-Amino-6-chloro-9-[2-(phosphonomethoxy)ethyl]purine was esterified with 3-hexadecyloxy-1-propanol (HDP) or 3-octadecyloxy-1-ethanol (ODE). Acidic hydrolysis yielded the new derivatives, HDP-PMEG and ODE-PMEG, which we screened for antiviral activity, *in vitro*. HDP-PMEG strongly inhibited viral replication in cells infected with HCMV (EC_{50} = 0.0002 μM), HSV-1(EC_{50} = 0.001 μM), HIV-1 (EC_{50} = 0.0008 μM), vaccinia (EC_{50} = 0.091 μM), and cowpox (EC_{50} = 0.12 μM). The esterified nucleotides proved significantly more potent than unmodified PMEG in all antiviral assays. These results suggest that long-chain PMEG alkoxyalkyl esters function as prodrugs of PMEG and may have potential for sustained local intravitreal delivery of this agent for antiviral or antiproliferative therapy of retinal diseases as well as having potential against poxvirus and herpesvirus diseases.

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Antiviral Activity of Cyclopentenyl Nucleosides Against Orthopox (Smallpox, Monkeypox and Cowpox), West Nile and Ebola Viruses

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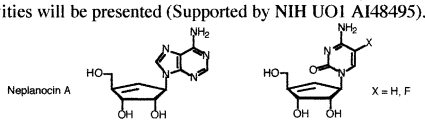
Carbocyclic nucleosides have received much attention, some of which exhibit potent biological activities. Since the isolation of aristeromycin and neplanocin A from natural sources, a number of carbocyclic nucleosides have been synthesized. For these carbocyclic nucleosides, L-cyclopent-2-enone serves as a key intermediate. However, the availability of this intermediate has been problematic due to low and inconsistent yields. Therefore, an efficient synthetic methodology for the key intermediate, L-cyclopent-2-enone is highly desirable for the synthesis of optically pure carbocyclic nucleosides. Here we reported an improved synthetic method for enantiomerically pure D-cyclopentenyl nucleosides using the key intermediate L-cyclopent-2-enone and their antiviral activities against Orthopox, West Nile and Ebola viruses. The key intermediate, L-cyclopent-2-enone, was prepared from D-ribose with ring closing metathesis in 8 steps. Coupling of (+)-cyclopentenyl alcohol with appropriately blocked purine and pyrimidine bases *via* Mitsunobu reaction followed by deprotection afforded the target D-cyclopentenyl nucleosides. Among the synthesized nucleosides, adenosine, cytosine and 5-F-cytosine analogs exhibited potent to moderate anti-Orthopox and anti-West Nile virus activities and 5-F-cytosine analog also showed anti-Ebola virus activity. Detailed chemistry and biological activities will be presented (Supported by NIH UO1 AI48495).



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Prevention of Poxvirus Infection by Tetrapyrroles

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Porphyrins and phthalocyanines have been found to be potent inhibitors of infection by vaccinia virus in cell culture. Previous studies have shown that these two types of tetrapyrroles can inhibit some viruses via photodynamic mechanisms. The current study, however, investigates light-independent inhibition activity. The Western Reserve (WR) and International Health Department-J (IHD-J) strains of vaccinia virus were used. Virucidal and antiviral activities as well as the cytotoxicity of test compounds were determined. Examples of active compounds include zinc protoporphyrin, copper hematoporphyrin, meso(2,6-dihydroxyphenyl) porphyrin, the sulfonated tetra-1-naphthyl and tetra-1-anthracenyl-porphyrins, selected sulfonated derivatives of halogenated tetraphenyl porphyrins, and the copper chelate of tetrasulfonated phthalocyanine. EC50 values for the most active compounds are as low as 0.05 ug/ml (40 nM). One of the most active compounds was the neutral meso(2,6-dihydroxyphenyl)porphyrin, indicating that the compounds do not have to be negatively charged to be active. These tetrapyrroles were found to be active against two different virus strains, indicating that these compounds may be broadly effective in their ability to prevent poxvirus infection.

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Efficacy of Single or Interval Dosing with Cidofovir Given Before or After Infection with Orthopoxviruses in Mice

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Orthopoxviruses, including variola and monkeypox, pose risks to human health through natural transmission or potential bioterrorist activities. Continued evaluation of effective antiviral agents is critical in preparation against an event of large scale exposure of susceptible populations to smallpox. We have utilized BALB/c mice inoculated intranasally with Vaccinia virus (VV) or Cowpox virus (CV) as models for systemic poxvirus infections and have previously reported on the efficacy of multiple doses of cidofovir (CDV). In the current studies, CDV was highly effective ($p<0.001$) in preventing mortality from VV when administered as a single injection of 100, 30 or 10 mg/kg 24h prior to or 24 or 72h after infection. A single dose of 100 mg/kg CDV was highly effective ($p<0.001$) when given 3 or 5 days prior to VV infection. Similar results were obtained against CV. Multiple interval treatments of CDV using 20, 6.7 or 2 mg/kg were evaluated against CV when treatments were initiated at 24, 48, or 72 h after infection. When given daily or every other day for 7 days, all treatment schedules significantly reduced mortality. When given every 3 days, CDV was effective at all doses beginning 24h after infection, but only the 20 and 6.7 mg/kg doses were effective if delayed until 48 or 72hrs. These data confirm the long lasting effect of CDV against poxvirus infections in mice and suggest that a single dose or 2-3 doses per week could be used for either prophylaxis or post-exposure treatment of smallpox in man.

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Activity of Cidofovir in a Murine Model for Cutaneous Vaccinia Virus Infection.

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Vaccination against smallpox with the vaccinia virus (VV) may cause severe skin lesions in individuals with an altered immune response or in patients with atopic dermatitis. We here report that athymic hairless (nu/nu) mice that have been scarified on the back and then inoculated in the scarified area with VV (Copenhagen strain) develop typical vaccinia lesions within 4 to 6 days post infection. The animals remain otherwise relatively healthy but about sixty % of them develop within 5 to 6 weeks post infection disseminated vaccinia lesions at every possible location on the surface of the body. Topical treatment with cidofovir (1% in DMSO) started on the day of infection completely prevented the development of cutaneous vaccinia lesions as well as the dissemination of the infection. Experiments are underway to determine whether cidofovir is also able to stop progression of the lesions if therapy is initiated at the time that cutaneous lesions have already appeared.

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Predictability of Genome Levels in Blood for Levels in Tissues of Cowpox Infected Mice

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Knowing the total viral burden can be a useful tool in disease management. Genome levels in blood may however be the only information readily obtainable from smallpox patients to evaluate the success of antiviral therapy. Studies with variola (the virus that causes smallpox) and monkeypox infected cynomolgus monkeys have shown that virus levels in blood are significantly reduced in monkeys successfully treated with cidofovir compared with controls. In order to begin to understand how the level of virus in blood correlates with virus levels in tissues we have utilized the uniformly lethal intranasal infected cowpox mouse model as a surrogate to correlate virus in blood and tissues throughout the course of this lethal infection. Quantitative MGB-Taqman PCR utilizing a pan-orthopox primer/probe set directed against the viral hemagglutinin gene allowed for evaluation of virus levels of 1000 gene copies per ml of whole blood or gram of tissue. Virus was first detectable in blood at 48 hours after infection and peaked at greater than 10^5 genomes per ml by day 5 post infection. Near real time monitoring of virus levels in blood may allow for rapid evaluation of the efficacy of antiviral therapy during an initial smallpox outbreak.

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Combinatorial antibodies against orthpoxviruses

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Concerns regarding planned destroying of smallpox virus collections and bioterrorism problem have fueled renewed interest in orthopoxviruses investigation. Development of antivirals against *Orthopoxviruses* is the important part of the investigation. The panels of human antibodies against orthopoxviruses could be a valuable resource because they could find application as immunotherapeutic or immunoprophylactic reagents, and as analytical reagents, in addition. Pannels of human scFv phage antibodies against *vaccinia virus, Elstree* and different strains of *variola virus*, have been obtained form a combinatorial phage library of human single-chain antibody fragments, scFv, (Medical Research Council Centre, Cambridge, England). Affinity and cross-reactivity of the selected antibodies in binding reaction with several orthopoxviruses including different strains of variola virus has been assayed using indirect ELISA. All selected antibodies have been tested for their capability to neutralize vaccinia virus and different strains of variola virus.

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Should the Guidelines for Revaccination Against Smallpox be Changed? Samuel Baron, Jingzhi Pan, and Joyce Poast, University of Texas Medical Branch, Dept. Microbiology & Immunology, Galveston, TX, US

Recent suggestions to revise current guidelines to delay the recommended ten-year revaccination may be based on insufficient and conflicting evidence of the persistence of immunity. The evidence that cell-mediated immunity and neutralizing antibody persists after one vaccination is conflicting. The levels are often low and their protective activity *in vivo* is unclear. *In vivo* reports of durable immunity to smallpox were not sufficiently controlled. Furthermore, shorter persistence of resistance to dermal challenge with the antigenically related vaccinia virus has been reported. To further examine persistence of neutralizing antibody to poxviruses, we compared neutralizing titers against vaccinia virus in sera from singly vaccinated persons, unvaccinated persons, and a normal commercial immunoglobulin (Panglobulin). We confirmed that the anti-vaccinia virus titers of sera from singly vaccinated adults are low (average 32 units/ml). The titer of 11 times concentrated normal commercial immunoglobulin was 150 U/ml, which confirms the low titer found in single vaccinated adults. Unexpectedly, the average titer of the unvaccinated subjects was 14 U/ml, which raises questions about the significance and specificity of residual antibody in vaccinees. To determine whether singly vaccinated sera are protective *in vivo*, mice were pretreated s.c. with 1 ml of either (1) serum from a single-vaccinated subject containing 10 U/ml neutralizing activity, (2) a single-vaccinated serum containing 43 U/ml, or (3) normal commercial immunoglobulin containing 150 U/ml, and challenged 24 hrs later i.p. with one LD_{100} vaccinia virus, strain IHD-E. The lowest titer serum (10 U/ml) did not protect the mice against lethal systemic infection, whereas the highest titer serum (43 U/ml) and the commercial immunoglobulin (150 units/ml) protected only 50% of the mice. In comparison, 100% protection occurred after treatment with 100 μg of the known interferon inducer, Poly I:CLC, i.m. Thus, our findings indicate that persistence of effective humoral antibody immunity to poxviruses remains questionable. More studies are needed before revising the recommended ten-year revaccination.

Oral Session V: Hepatitis C Virus, Flaviviruses

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Structure Activity Relationship of 2' Modified Nucleosides for Inhibition of Hepatitis C Virus

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Hepatitis C virus (HCV) is the pathogen associated with the majority of sporadic and transfusion related non-A and non-B hepatitis infections. While often asymptomatic, HCV infection can progress to chronic hepatitis, sometimes leading to liver cirrhosis and hepatocellular carcinoma. Current estimates suggest that 170 million people worldwide suffer from HCV infection. Treatment options comprise the use of recombinant interferon-α in combination with ribavirin. However, the clinical benefit is limited, resulting in a sustained antiviral response in about 50% of the recipients. Moreover, a vaccine has not yet been developed. Hence HCV infection represents a significant health problem and has become the focus of intense investigations in order to identify effective therapies

HCV encodes for a series of non-structural proteins, NS2 through NS5B, several of which are considered essential to HCV replication and proliferation, and hence represent valid candidates for therapeutic intervention. The RNA dependent RNA polymerase, NS5B, encoded at the C-terminal portion of the HCV protein, displays significant structural divergence from cellular polymerases, and is an obvious target for intervention by design of specific inhibitors.

In order to identify lead compounds for further SAR on HCV RdRp, a series of purine ribo nucleoside analogs was evaluated for anti HCV activity and two 2'modified ribo nucleosides identified as potent inhibitors of HCV RNA replication in vitro. For active nucleosides, identity of target was confirmed at the triphosphate level on the purified WT NS5BΔ55. We describe here the SAR of a series of related 2'modified ribonucleosides; including introduction of modified bases, various substitutions, and change of stereo- and regio-chemistry on the sugar moieties.

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Synthesis and Pharmacokinetic Properties of Nucleoside Analogues as Possible Inhibitors of HCV RNA Replication.

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The number of individuals infected with hepatitis C virus worldwide is currently estimated at 170 million and continues to increase, with serious sequel including cirrhosis and hepatocellular carcinoma. Also, a significant number of patients are coinfected with HIV, which is associated with a more rapid disease progression. Current treatments for HCV infection include a combination of ribavirin and interferon-alpha, which leads to viral clearance in about half of treated patients. The need for more efficacious treatments has necessitated drug discovery efforts based on antiviral targets including the virally-encoded protease NS3, the viral IRES, and antisense approaches. We have targeted the HCV RNA-dependent RNA polymerase, an essential enzyme for HCV RNA replication, using ribonucleoside analogs. We have synthesized a large number of diverse molecules and have found several 2'-modified nucleosides that demonstrate potent inhibitory activity in a cell-based replicon assay. Some of the compounds display promising pharmacokinetic properties *in vivo*. We have made a series of compounds to investigate structure-activity relationships. Synthetic strategies and biological data will be presented.

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2'-Modified Nucleoside Analogs as Inhibitors of Hepatitis C RNA Replication D. B. Olsen*, B. Bhat** , M. Bosserman*, S. S. Carroll*, L. Colwell^, R. De Francesco^^, A. B. Eldrup** , O. Flores*, K. Getty*, R. LaFemina*, M. MacCoss^, G. Migliaccio^^, A. L. Simcoe*, C. A. Rutkowski*, M. W. Stahlhut*, J. E. Tomassini*, B. Wolanski* *Department of Biological Chemistry, Merck Research Laboratories, West Point, PA, 19486, **Isis Pharmaceuticals, Carlsbad, CA, 92008, ^Department of Medicinal Chemistry, Merck Research Laboratories, Rahway, NJ, 07065, ^^Istituto di Ricerche di Biologia Molecolare, P. Angeletti, 00040 Pomezia -(Roma), Italy

Chemotherapies for the treatment of hepatitis C virus (HCV) infection consist of combinations of interferon alpha and ribavirin which are efficacious in achieving a sustained viral response in ca. 40-60% of patients, but improved therapies are needed. The success of antiviral chemotherapies based on administration ofanalogs of deoxyribo-nucleosides as treatment for HIV, herpes virus, and hepatitis B infection prompted a focussed screening effort to identify nucleoside analogs that inhibit HCV replication. Nucleoside analogs modified at the 2'-position were found to inhibit the synthesis of viral RNA in a cell-based replicon assay in the absence of cytotoxicity. The corresponding 5'-triphosphates were found to inhibit RNA synthesis catalyzed by HCV RNA polymerase. Gel-based incorporation assays indicated that the RNA polymerase is capable of incorporating these analogs on to a growing RNA strand. However, after incorporation of the analogs, the addition of the next nucleotide is significantly impaired, suggesting that, although these analogs retain a 3'-hydroxyl, the enzyme is not able to utilize it efficiently to form the phosphodiester bond. Resistance to inhibition by the 2'-modified nucleosides is imparted by an active site mutation in the RNA polymerase. In cell culture, the 5'-triphosphates of these nucleosides are detected intracellularly following addition of the nucleosides to the media. Pharmacokinetic parameters determined for these nucleosides indicate that only one achieves a significant concentration in rat plasma after oral dosing. The antiviral specificity of these nucleosides has been determined and the results suggest decreasing antiviral potency as the virus becomes more distantly related to HCV.

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Enhancement of Activity Against HCV Replication by Combination Therapy in the HCV Replicon Model B. Korba[1], A. Ciancio[2], C. Okuse[3], and J. Rinaudo[1]. 1-Georgetown Univ., Rockville, MD USA 2-Dept. of Gastroenterology, Ospedale S. Giovanni Battista di Torino, Italy 3-Dept. of Internal Med., St. Marianna Univ. School of Medicine, Kawasaki, Japan

Alpha, beta, and gamma interferons have been shown to be effective inhibitors of HCV replication cultured cells carrying HCV replicons. To help define the divergent cellular processes involved in the control of intracellular HCV replication, we have characterized the activity of monotherapy and combination therapy with the major types of human interferons against HCV replication in the HCV replicon-containing cell line, AVA5. Alpha, beta, and omega were approximately equally effective at inhibiting HCV replication, while gamma was approximately 10-fold more potent. The combination interferon therapies produced enhanced anti-HCV activity in many cases, and displayed diverse types of interactions and HCV inhibition kinetics. Alpha and beta acted in a generally additive manner, alpha and omega were strongly antagonistic, and alpha/gamma combinations displayed the most enhanced, strongly synergistic antiviral effects. Microarray analysis revealed distinct, treatment-related differences in gene expression patterns. AVA5 cells were capable of mimicking some of the gene expression patterns associated with acute HCV infection in chimpanzees. Alpha uniquely resulted in the down-regulation of 10 genes, but beta up-regulated only 2 genes not affected by any other treatment. Interferon gamma and omega treatments uniquely induced 24 and 25 genes, down-regulating only 1 and 3 genes, respectively. Transcriptional activators/inhibitors, RNA-binding proteins, cell cycle, apoptosis, and signal transduction factors were some of the classes of genes affected by the antiviral treatments. Multi-dimensional Scaling analysis revealed that the overall gene expression patterns under alpha and beta interferon monotherapy were highly similar, and very divergent from that under alpha/gamma combination therapy. We are currently utilizing the monotherapy gene expression patterns to develop mechanisms to account for the types of interactions observed during combination therapy. Interferon therapy can be effectively modeled using HCV replicon technology yielding potentially more effective treatment regimens. Combinations with other interferons can significantly enhance the anti-HCV activity of alpha interferon.

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A Cell-Based Assay for Evaluation of Anti-Viral Agents against HCV

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In vitro cultures of cell lines capable of supporting HCV propagation are critical for understanding viral replication and pathogenesis, as well as for evaluating anti-HCV compounds. One of the schemes currently used for supporting HCV replications is the replicon transfection-based system. Our approach for developing a more natural and biologically relevant system was to infect human cell-lines with HCV derived from HCV patients. The requirements from this system were to clearly demonstrate viral infection of the cells, viral replication, emergence of viral particles, and ability to use the system for testing the efficacy of potential therapeutics. Methods: Human hepatoma cell lines were infected with HCV by incubation with HCV-positive serum from HCV patients. Cells were sub-cultured weekly and viral infection was followed by detection of HCV-RNA in cells and in culture supernatant. HCV infection of the cells was further confirmed by in-cell in situ hybridization. Viral replication was assayed by detection of (-) strand RNA in infected cells. Results: HCV-RNA could be detected in cells up to four weeks post infection. It could be detected in supernatants after a week-long culture and up to three weeks post infection. The presence of HCV-RNA in the supernatants of infected cultures could indicate the emergence of viral particles. The existence of HCV infectious particles in cell cultures is supported by preliminary data showing ability to re-infect new cells. Presence of (-) strand RNA was observed in cells but not in cultured supernatant. Feasibility of using this cell-based system for evaluating efficacy of anti-viral agents was tested using IFN-alpha as well as human monoclonal antibodies directed against the envelope protein of HCV. Treatment of infected cells with IFN-alpha resulted in significant reduction of HCV-RNA levels in cells. In addition, human monoclonal antibody against E2 was able to inhibit HCV infection when pre-incubated with the infectious serum. Conclusions: Thus, our cell culture system supports viral infection and viral replication in human hepatoma cells. This system could be adapted as a cell-based assay for evaluating potential antiviral agents.

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Discovery of a novel class of HCV NS5B RNA dependent RNA polymerase inhibitors: SAR studies and activity in replicon cells.
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A functional hepatitis C virus (HCV) RNA-dependent RNA polymerase has been shown to be critical for viral replication; thus this enzyme represents an attractive target for the discovery of novel antivirals. A polymerase assay using a full-length recombinant enzyme expressed from baculovirus-infected Sf9 insect cells was developed. Activity was determined using a FlashPlate™ scintillation proximity assay to measure the incorporation of radiolabelled UTP in a polyrA/ biotinylated-oligo dT template-primer captured on the surface of streptavidin-coated microtiter plate. Our screening campaign resulted in the identification of a novel class of substituted 3-amino thiophene derivatives as potent inhibitors of NS5B polymerase. Structure activity relationship studies (SAR) have delineated the optimum substitution pattern for activity, for example, a carboxylic acid at the 2-position of the thiophene scaffold is necessary for activity. Structures of various polymerase-inhibitor complexes were also elucidated by soaking experiments and this led to the identification of an allosteric binding site within the thumb region about 30 Å from the active site. The ability to inhibit replication of viral subgenomic RNA (replicon) in Huh-7 was determined using the system developed by Bartenschlager. One of the best compounds of this study had the following profile: NS5B polymerase IC_{50} 0.74 μM; viral replication IC_{50} 0.24 μM (reduction of viral RNA was quantified by PCR RNA analysis); Huh-7 CC_{50} 105 μM (measured by incorporation of ^{3}H-thymidine). In summary, a novel class of potent inhibitors of HCV NS5B mediated subgenomic viral replication was identified and the profile of these compounds warranted further evaluation as preclinical candidates.

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The Trimera Model: A Tool for Differential Gene Expression Analysis in HCV-Infected Human Liver Cells

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The molecular mechanisms by which HCV infects human liver cells, induces liver damage and promotes hepatocellular carcinoma are still poorly understood. This is due, mainly, to the lack of a reliable HCV model. Recently, a model for hepatitis C virus (HCV) infection, namely the HCV-Trimera model, was described. This model involves HCV-infected human liver fragments transplanted into a normal strain of mice. In this study, the HCV Trimera model is employed for the analysis of differential gene expression in the process of HCV infection of human liver. Such a model allows us to compare gene expression profiles between infected and non-infected identical human tissue thereby maintaining the same genetic background. Normal human liver fragments were infected with normal or HCV-positve serum and then transplanted under the kidney capsule of immunosupressed Trimera mice. At given time points the human liver grafts were removed, total RNA extracted and suitably labeled. This RNA was used for hybridization to the Affymetrix Human Genome U95 microarray. Gene expression profiles from HCV-infected and non-infected liver grafts were compared 3 days after infection. Gene expression changes of 3.5 fold or greater were detected for 707 genes. 512 genes being up-regulated and 195 down-regulated in the HCV-infected samples. Of these genes, 42 exhibited changes of over 10-fold. These include IFN-response genes, MHC molecules, cytoskeletal proteins, proteasome components, cytokines, growth factors, cell cycle regulators and signal transduction genes. The genes are currently under further analyses. This study indicates that the Trimera Model is a practical tool for studying gene expression during the process of HCV infection and replication. These observations could potentially lead to elucidating molecular mechanisms and alterations triggered by HCV and to the discovery of new therapeutic targets.

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Evaluation of therpeutics against West Nile virus in a hamster model. C. W. Day, J. D. Morrey, R. W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, Utah, USA.

Golden Syrian hamsters were used to evaluate candidate therapeutic substances against the New York strain of West Nile virus. The virology and disease pathogenesis was compared with infection in mice to determine that hamsters displayed signs of disease (mild fever, transient viremia, abrupt onset, muscle weakness, imbalance, somnolence, tremors in extremities, paresis, unresponsiveness and death) similar to those observed in human patients. Different strains of West Nile virus conferred different degrees of pathogenesis in the hamster model. Ribavirin, imiquimod™, ampligen™ and other candidate compounds were evaluated in the hamster model. When administered 4 hours before intraperitoneal (i.p.) viral challenge, ribavirin did not affect mortality. Topical imiquimod once daily and i.p. amligen every other day, however, did have some beneficial effect on survival. In conclusion, the West Nile virus hamster model will provide beneficial information in planning future clinical trials and in the treatment of West Nile virus infection. (Supported by Contract NO1-AI-15435, NIAID, NIH)

Oral Session VI: Poxviruses

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Cidofovir Treatment of Variola (Smallpox) in the Hemorrhagic Smallpox Primate Model and the IV Monkeypox Primate Model

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Cidofovir is capable of inhibiting smallpox and other orthopoxviruses *in vitro* and can successfully treat mice that are lethally infected with cowpox by either the intranasal or true small particle aerosol routes. Aerosol infection of cynomolgus monkeys with high doses (10^7 pfu) of the Zaire 76 strain of monkeypox produced a lethal fibrinonecrotic bronchopneumonia, which could be successfully treated with cidofovir. To access the efficacy of cidofovir against smallpox, two models of intravenous infection of cynomolgus monkeys with variola (smallpox) were developed based on challenge dose. The extent of the characteristic pox rash of smallpox and monkeypox correlates with disease severity in man. Intravenous infection with 10^7 pfu of monkeypox produces a lethal model that faithfully reproduces the rash lesional disease characteristic of smallpox and monkeypox, while similar studies with 10^8 pfu the Harper strain of variola produced a similar lesional disease with > 250 lesions (WHO category "grave") and 33% mortality (day 11), but increasing the virus challenge dose 10-fold (2 X 10^{10} genomes) resulted in a 100% acutely lethal disease (MTD 4 days) that more closely mimics hemorrhagic smallpox, with virus levels in organs 1,000 to 10,000 fold greater. This model of hemorrhagic smallpox was used to demonstrate successful prophylaxis with cidofovir, but the overwhelming nature of the infection makes the hemorrhagic smallpox model inappropriate to determine the treatment window for classical smallpox. To determine if a lesional model could be successfully treated, we use the uniformly lethal IV monkeypox model that corresponds more closely to the 10^8pfu of variola model to show that cidofovir prophylaxis provided complete protection, showed no signs of illness and controlled virus replication in blood, while the placebo-treated animal had >850 lesions and levels of virus in blood > 10^7 genomes/ ml and died on day 12. Based on these studies we believe the lesional model of smallpox produced by 10^8 pfu of Harper strain is the most appropriate primate model for drug evaluation.

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Aerosolized Cidofovir is Retained in the Lung and Protects Mice Against Lethal Intranasal Cowpox Virus Infection

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We previously showed that a low dose of aerosolized cidofovir (HPMPC, Vistide®) protected mice against aerosolized cowpox virus more effectively than a much larger dose of the same drug given subcutaneously, suggesting that aerosolized cidofovir is retained in the lung. We now report the results of a pharmacokinetic study comparing the tissue distribution in mice of ^{14}C-cidofovir delivered by aerosol or subcutaneously. Aerosol treatment resulted in a high concentration of cidofovir in lung tissues at 1 hour postexposure that remained markedly elevated over background 96 hours postexposure. Little drug was detected in the kidneys 1 hour post-exposure. In striking contrast, subcutaneous administration resulted in a low level of cidofovir in the lungs and a rapid development of a high concentration in the kidneys. Because nephrotoxicity is a major concern in cidofovir therapy, the results indicate that the direct delivery of cidofovir to the respiratory tract is an effective treatment strategy that maximizes the tissue concentration of the drug at the initial site of viral replication, while minimizing its accumulation in the kidneys. As further evidence of the protective efficacy of aerosolized cidofovir against lethal orthopoxvirus infection of the respiratory tract, we found that aerosol-treated mice were protected against cowpox virus introduced by intranasally. All or nearly all mice that were treated once by aerosol, from 2 days before to 2 days after challenge, survived intranasal inoculation, while all placebo-treated animals died.

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Vaccinia Skin Lesions in Immunosuppressed Hairless Mice can be Treated Topically but not Parenterally with Cidofovir.
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We developed a new animal model in which immunosuppressed (using cyclophosphamide) hairless mice exhibit disseminated disease and later die following the infection of two cutaneous sites (hip and shoulder) with the WR strain of vaccinia virus. Severely infected animals developed greatly expanded primary lesions and numerous satellite lesions on other areas of the body. Intraperitoneal (i.p.) treatments with cidofovir (100 mg/kg/day) every three days for 21 days had little impact on the disease, and high virus titers were present in primary lesions, suggesting that the drug penetrated poorly into the skin. Although the virus eventually spread internally into visceral organs such as the lungs and liver, virus titers were insufficiently high in placebo-treated animals to account for death. Parenteral cidofovir treatments reduced visceral organ titers but the mice died anyway. Topical treatments with cidofovir were given using a cream-based formulation applied twice daily for 7 days starting 1, 3, or 5 days after infection. Primary lesion development and satellite lesion formation were maximally suppressed from treatments initiated on day 1. Cidofovir concentrations of 0.5, 1, and 3% produced dose-responsive effects on lesion severity and survival. A positive effect on these parameters occurred even when treatments started 9 days post-virus exposure, which was well after primary lesions had appeared. Lesion size reduction was accompanied by reduction in lesion virus titers. The results suggest that disseminated virus replication in the skin causes death. The failure of parenterally-administered cidofovir in this infection model implies that smallpox in humans may have to be treated topically. We hypothesize that treatment failure in early smallpox trials in humans with then-existing antiviral drugs may have been because they were systemically administered and did not reach the sites of infection in sufficient concentration, and that some of these compounds may be effective if applied topically. [Supported by contract NO1-AI-15435 from the Virology Branch, NIAID, NIH]

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Effect of Oral Treatment with Alkoxyalkyl Esters of Cidofovir on Cowpox or Vaccinia Virus Infections in Mice
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The efficacy of cidofovir (CDV) against orthopoxvirus infections has been well documented, however, the drug is not active orally and has significant adverse effects. Four alkoxyalkyl esters of CDV were evaluated for efficacy and toxicity in mice inoculated intranasally with cowpox virus (CV) or vaccinia virus (VV). 1-O-hexadecyloxypropyl-CDV (HDP-CDV), 1-O-octadecyloxyethyl-CDV (ODE-CDV), 1-O-oleyloxypropyl-CDV (OLP-CDV) or 1-O-oleyloxyethyl-CDV (OLE-CDV) were prepared in water and administered once daily by oral gavage to mice using 20, 6.7 or 2 mg/kg or 10, 7.5, 5, 2.5, or 1.25 mg/kg for 5 days beginning 24, 48 or 72 hrs post infection. HDP-CDV, ODE-CDV or OLE-CDV were effective at preventing mortality to CV at 24 hr, but HDP-CDV and ODE-CDV maintained efficacy when begun at 72 hr. Pretreatment with HDP-CDV or ODE-CDV was also effective beginning at 5, 3 or 1 day prior to CV inoculation. ODE-CDV or OLE-CDV were also effective against VV infections at 24 or 48 hr after infection. These data suggested that HDP-CDV and ODE-CDV were the most promising candidates of the 4 analogs evaluated. Based on these data and pharmacokinetic profiles, alternative dosing regimens of HDP-CDV or ODE-CDV against CV or VV will provide additional information on the potential of these orally active CDV analogs for treatment of human orthopoxvirus infection.

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Effect of Oral Ether Lipid Analogs of Cidofovir on Mortality and Viral Infectivity Levels in Tissues in a Lethal Ectromelia Virus Challenge Model
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Alkoxyalkanol esters of cidofovir (CDV) have been synthesized and evaluated against poxvirus replication *in vitro*. The modified CDV analogs have >100 fold more antiviral activity than unmodified CDV against cowpox, vaccinia and variola virus infected cells. These compounds, 1-O-hexadecyloxypropyl-CDV (HDP-CDV) and 1-O-octadecyloxyethyl-CDV (ODE-CDV), are orally bioavailable in mice. In this study, we compared the oral activity of HDP-CDV, ODE-CDV, 1-O-oleyloxypropyl-CDV (OLP-CDV) and 1-O-oleyloxyethyl-CDV (OLE-CDV) with oral CDV in A/J mice. Mice were infected with 2 x 10^6 PFU by aerosol and 4 hours later oral treatment was begun with 30, 10 and 3 mg/kg of the drugs once daily by gavage for 5 days. At 30, 10, and 3 mg/kg of oral CDV, 82%, 100%, and 100% of challenged mice die. >90% survival was obtained with 30 or 10 mg/kg of HDP-CDV, OLP-CDV and OLE-CDV. >90% survival was also obtained with 10 or 3 mg/kg ODE-CDV. Effective oral doses of the CDV ether lipid analogs also reduced morbidity as measured by a grading scale. Control viral infectivity levels in spleen and liver ranged from 2 to 9 x 10^6 PFU/ml and 0.5 to 2 x 10^7 PFU/ml, respectively. Oral doses of the CDV analogs which reduced mortality to <10% reduced viral infectivity levels to <1 x 10^2 in spleen and liver (i.e. below the limit of detection). In effectively treated animals, lung virus infectivity levels were generally less than in controls, but significant levels of viral infectivity in lung remained. ODE-CDV appeared to be the most potent analog with HDP-CDV, OLP-CDV and OLE-CDV having similar antiviral activity against a lethal challenge with ectromelia virus *in vivo*. HDP-CDV and ODE-CDV are worthy of further evaluation as a potential oral therapy for poxvirus infections.

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Oral Pharmacokinetics and Tissue Distribution of 1-O-Hexadecyloxypropyl-[2-^{14}C]Cyclic Cidofovir in Mice
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It has been been reported previously that 1-O-hexadecyloxypropyl-cidofovir (HDP-CDV) and HDP-cyclic cidofovir (HDP-cCDV) exhibit multiple log increases in antiviral activity versus CDV against human cytomegalovirus (HCMV) and against orthopoxviruses such as cowpox, monkeypox, vaccinia and smallpox. HDP-CDV has also been shown to have 93% oral bioavailability versus <5% for CDV. In this study, we examined the oral bioavailability of HDP-cCDV in mice and made some comparisons with intraperitoneal (ip) ^{14}C-CDV. Plasma levels of drug were evaluated after a single oral or subcutaneous (sc) dose of 10 mg/kg ^{14}C-labeled HDP-cCDV to mice. Blood samples were obtained from 3 separate animals at 30 minutes and at 1, 2, 3, 6, 12 and 24 hours. Aliquots of plasma were counted for each time point and drug content was determined. Spleen, kidney, lung, liver, heart, and brain were also obtained and the organs were weighed in vials and treated with TS-2 tissue solubilizer and counted by liquid scintillation. Peak plasma levels of HDP-cCDV were 0.88 μM at 30 min (sc) compared to 1.10 μM (oral) at 2 hr each declining to 0.03 μM at 24 hr. Compared to an equimolar dose of ^{14}C-labeled CDV given ip, oral HDP-cCDV gave peak kidney levels of 4.24 nmol/gm versus 180 nmol/gm with ip CDV. In summary, HDP-cCDV is orally bioavailable and provides plasma and tissue levels of drug sufficient to inhibit replication of HCMV, vaccinia, cowpox, monkeypox and variola viruses. Alkoxyalkyl esters of cyclic cidofovir are worthy of further investigation in animal models of CMV and poxvirus diseases.

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Development of a rodent model for lethal monkeypox infections and its use in antiviral drug testing

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Poxviruses are viruses that pose a significant threat to humans in the context of biological warfare or biological terrorism. The development of antiviral drugs that inhibit these viruses is of the highest priority. Variola, the causative agent of smallpox, only naturally infects humans and can be made to infect non-human primates in a laboratory setting. However, work with both variola in biosafety level 4 as well as non-human primates is both expensive and dangerous. Monkeypox virus produces a disease in humans and monkeys that is indistinguishable from smallpox. We have thus established a rodent model of lethal monkeypox infection using cotton rats (*Sigmodon hispidus*). Monkeypox-Zaire virus was used to challenge weanling cotton rats by both the intranasal and intraperitoneal routes. LD_{50} values were approximately $1x10^6$ and $1x10^5$ pfu/aminal for i.n. and i.p. routes respectively. We subsequently used this model to test the antiviral efficacy of subcutaneously inoculated cidofovir and have been able to protect lethally challenged animals. We have used a LightCycler-based real-time PCR assay to track virus levels in cotton rat tissues and blood in both drug-treated and placebo-treated animals. Finally, we have examined the pathology of major target organs of virus infection. We conclude that the cotton rat model of monkeypox infection is an appropriate surrogate for non-human primate models or orthopox virus infections, and that it can be used to pre-screen antiviral drugs for further development and testing.

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Smallpox Model: Protection by IFN and Poly I:CLC Despite Evasive Mechanisms. Baron, Samuel[1], Salazar, Andres[2], Pestka, Sidney[3,4], Poast, Joyce[1], and Clark, Bill[3]. [1]University of Texas Medical Branch, Galveston, TX USA; [2]Ribopharm, Inc., Washington, DC, USA; [3]PBL Biomedical Laboratories, Piscataway, NJ, [4]Robert Wood Johnson Medical School-UMDNJ, Piscataway, NJ, USA.

A bioterrorist attack using smallpox may occur. Susceptibility of our population is high due to waning immunity. IFN and inducer treatment has the potential to protect against smallpox. Previous reports show protection against poxviruses even though *in vitro* poxviruses are resistant and have mechanisms to evade IFN. In our *in vitro* experiments, cultured mouse L cells infected with the smallpox-related vaccinia virus were 20 to 100-fold less sensitive to the three IFNs than was the IFN-sensitive vesicular stomatitis virus. In contrast, *in vivo,* mice infected i.p. and treated with recombinant Mu-IFN-αA or the INF inducer Poly I:CLC were 80-100% protected against mortality due to disseminated vaccinia virus. The minimum protective i.m. dose of IFN was 65,000 U/mouse. The minimum protective i.m. dose of the IFN/cytokine-inducer Poly I:CLC was 3 μg/mouse. Protection by Poly I:CLC occurred when it was administered as a single dose starting 8 days prior to infection, through 6 hours post-infection. Greater than 100-fold inhibition of vaccinia virus multiplication occurred in spleens, livers, and lungs of mice treated with Poly I:CLC. I.M. Poly I:CLC, given at the same time as dermal vaccination did not prevent development of the dermal lesion and did not diminish production of neutralizing antibody. These findings show that in a mouse model of disseminated poxvirus infection, the interferon-inducer, Poly I:CLC and IFN strongly and rapidly protect against pox virus multiplication, morbidity and mortality *in vivo* despite the *in vitro* insensitivity and evasive mechanisms. The mechanism for *in vivo* protection may be the occurrence of cell types *in vivo* that do not respond to the evasive mechanisms of poxviruses. We may infer that Poly I:CLC and IFN given i.m. at the same time as vaccination of humans may provide immediate protection against smallpox, and may reduce vaccine complications, while not inhibiting the immune response to vaccination. IFN is available as a licensed pharmaceutical and Poly I:CLC is available as an investigational drug.

Poster Session III: Respiratory Viruses, Flaviviruses, Other Viruses

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Inhibition of clinically important respiratory viruses by beta-D-N^4-hydroxycytidine (NHC). Barnard, D.L.[1], Stowell, V.D.[1], Smee, D.F.[1] Sidwell, R.W.[1], Watanabe, K.[2], Stuyver, L.J. and Otto. M.J.[2] [1]Institute for Antiviral Research, Utah State University, Logan, UT, USA and [2]Pharmasset, Inc., Tucker, GA, USA.

Human respiratory viruses cause significant global morbidity and mortality as the result of severe, chronic, or often lethal disease. To develop effective therapies for treatment of these infections, beta-D-N^4-hydroxycytidine was synthesized and evaluated for efficacy against viruses causing clinically important human respiratory disease. In viral cytopathic inhibition assays, NHC inhibited influenza A and B viruses with EC_{50} (50% effective concentration) values from 0.5-19 μM. Measles virus strain Chicago (MV) was inhibited at 1 μM, respiratory syncytial virus strain A2 (RSV A2) at 0.5 μM and rhinovirus (RV) type 2 (strain HGP) from 20-30 μM; adenovirus type 1 (strain 65089/Chicago) and parainfluenza type 3 (strain 14702) were not inhibited. The antiviral activity of NHC was verified by virus yield reduction assays; the EC_{90} values ranged from 0.4 μM for influenza A (H1N1) strain Beijing/262/95 to 2 μM for RV 2 and RSV A2. It was not virucidal at concentrations inhibiting virus replication. NHC was not toxic (IC_{50} > 500 μM, 50% inhibitory concentration) in confluent cells such as Madin-Darby canine kidney (MDCK), human lung carcinoma (A549) and human oral epidermoid carcinoma cells (KB), but was slightly to moderately toxic in cells derived from African green kidney (MA-104 and BS-C-1); IC_{50} = 15-500 μM. In actively growing cells, IC_{50} values ranged from 21-33 μM. A preliminary study done in an influenza A (Shandong/09/93, H3N2) mouse model confirmed the in vitro efficacy. Using a 3-dose, intraperitoneal injection regimen (100, 50, 25 mg/kg/day for 5 days, BID), lung consolidation and lung scores for the 100 mg/kg/day group were reduced by 50% accompanied by inhibition of lung weight increases. Virus titers (day 9) were about one $\log_{10}$ less than in infected controls. Three of 10 animals survived the infection, which was not significant compared to the placebo-treated virus group. Animal toxicity consisted of slight weight loss. Thus, these data suggest that NHC should be further evaluated in animal models for efficacy against human respiratory virus infections.

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Structural Analysis of the Activation of Ribavirin Analogs by NDP kinase : Comparison with other Ribavirin Targets

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Ribavirin used in therapies against HCV is potentially efficient against several virus but presents a high cytotoxicity. Several ribavirin triphosphate analogs modified on the ribose moiety are synthesized and their specificity on several enzymes targeted by ribavirin are studied. The modified nucleosides and nucleotides include 2'-deoxy, 3'-deoxy, 2',3'-deoxy, 2',3'-dideoxy-2',3'-didehydro, 2',3'-epoxyribavirin derivatives. They are analyzed as substrates for NDP kinase responsible for the last step of cellular activation, as well as inhibitors for polymerases such as HIV-1 reverse transcriptase, T7 RNA polymerase, and HCV polymerase. X-ray structure of NDP kinase complexed with ribavirin triphosphate provides an explanation for its high reactivity and the crucial role of the 3'hydroxyl group. The 2'hydroxyl group is found important in HCV polymerase inhibition.

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ANTI-INFLUENZA AND ANTI-HERPETIC ACITIVITY OF DECAMETOXIN

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We have studied the efficacy of decametoxin antiviral action towards influenza and herpes. Decametoxin - [1, 10 - decamethylenbis(N, N dimethylmenthoxycarbonilmethyl)ammoniumdichloride] is the bis-quarternary ammonium-base(salt) contains L-menthol. This preparattion has passed all needed testing and was approved in Ukraine as an effective anti-septic with cationic surfactant action. It has demonstrated bacteriostatic and bactericidal action with wide antimicrobial and antimycotical activity spectrum. Antiinfluenza action of decametoxin was studied on influenza strains A/PR/8/34 and B/Leningrad/17/86. These viruses were reproduced on tissue culture of chorioallantoic membranes of chicken embryos. It was demonstrated that decametoxin taken in 25 mkg/ml dose has hindered in a statistically reliable manner the reproduction of influenza viruses A and B. We have shown experimentally that decametoxin hinders also the reproduction of herpes simplex virus (HSV-1) strain US in tissue culture Hep-2. This preparation taken in doses of 2 mkg/ml and 4 mkg/ml causes the decrease of virus specific intracnuclear inclusions in sensitive Hep-2 cells by 27 % and 60 % , respectively. Decametoxin even in minimal doses of 0.1 % causes the infectious virus titres in mice lungs on the fifth day since infection in experimental animal influenza. The preparation was introduced intranasally by clinical-prophylactic and clinical schemes. The application of decametoxin by clinical scheme decreases lethality of infected animals by 1.5 log LD_{50}. The use of the preparation by clinical-prophylactic scheme causes the lethality decrease by 2.0 log LD_{50}. The results of the present study allow us to state that decametoxin demonstrates sufficient antiviral activity and could be recommended for clinical trial.

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Biologically active constituents of a polyphenol extract from *Geranium sanguineum* L. with anti-influenza virus activity

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From the aerial roots of *Geranium sanguineum* L. a MeOH extract with strong antiviral activity has been isolated (Serkedjieva and Manolova, 1992). To investigate its active fractions, the extract was partitioned with solvents with increasing polarity. The n-BuOH fraction was shown to contain the majority of the *in vitro* antiviral activity and the EtOAc fraction - the majority of the *in vivo* protective activity. Further bioassay - directed fractionation of the $CHCl_3$, EtOAc and n-BuOH fractions was performed in order to obtain information about the active principles the antiviral effect. The individual constituents were identified by spectroscopic methods and comparison with authentic samples. The cell-toxic and virus-inhibitory effects were studied using the reproduction of representative influenza viruses in CEF cells. The results indicate that the virus inhibitory effect of the extract could not be accounted for simply in terms of polyphenol content neither could be attributed to one or few separate ingredients. The presence of a variety of biologically active compounds as well as the possible synergistic interactions between them seemed to be more significant for the overall antiviral effect of the plant preparation.

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Protease-inhibitory activity of a plant preparation with anti-influenza virus effect

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A MeOH extract from the medicinal plant *Geranium sanguineum* L. (PC) inhibited the reproduction of influenza A and B viruses in cell cultures and protected mice in experimental influenza infection (Serkedjieva and Manolova, 1992); the n-BuOH fraction was shown to contain the majority of the *in vitro* antiviral activity and the EtOAc fraction - the majority of the *in vivo* protective activity. We presumed that the protection might be attributed partially to a protease-inhibitory effect, known for the polyphenolic substances, present in PC. The protease-inhibitory activity of the total MeOH extract (PC) and its n-BuOH and EtOAc fractions was studied in a model system. For comparative reasons referent inhibitors of serine proteases were used. It was found that the total extract and the EtOAc fraction inhibited the activity of trypsin in a dose-dependent manner, while the n-BuOH fraction was inactive in the model system used. The enzyme-inhibitory activity of all three preparations correlated with the rate of the protective effect shown in the murine model of influenza virus A/Aichi/2/68 (H3N2) infection.

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Effect of Cranberry Juice Constituents on haeamgglutination and infectivity of Influenza Virus

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Cranberry juice contains high molecular weight inhibitors (NDM) of bacterial adhesion to host cells as well as of coaggregation of oral bacteria. Prevention of urinary tract infection in humans consuming cranberry is likely to be due to these adhesion inhibitors. Because the anti-adhesion activity of cranberry is broad spectrum, we sought to explore their potential for inhibiting viral adsorption to cells. The virus, a major cause of disease in children, medically compromised patients and the elderly, is known to express sialic acid specific heamagglutinin which mediates binding to tissue culture cells and heamagglutination of human red blood cells. Hemagglutination caused by both A (H1N1 and H3N2) and B virus strains was inhibited by NDM at concentration of 125 µg/ml which is ten fold lower than that normally found in cranberry juice cocktail. Viral infectivity was also decreased by NDM as evident by CPE reduction in MDCK cell monolayer. Virus preincubation with NDM reduced titer of virus from 10^7 to 10^1 whereas simultaneous addition of NDM and virus decreased the titer from 10^6 to 10^1.

NDM exhibit an in vitro inhibitory effect on influenza virus adsorption and CPE.

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Elaboration and Usage of 4D-QSAR Approach for Successful Antiviral Development

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In this research we investigated the influence of molecular structure of macrocyclic pyridinophanes and their analogs on their anti-influenza and antiherpetic activity. This goal was achieved by using the elaborated 4D-QSAR approaches on basis of simplex representation of molecular structure. Such representation for biologically active substances allows us to unify the description of spatial structure of compounds with saving of the complete stereochemical information. It allows us to easily determine common fragments of spatial structure either increasing or decreasing the concrete biological activity for researched molecules. Based on such approaches it is easy to realize molecular design of compounds with the given level of activity with the help of generation of the allowed combinations of such types simplexes, which determine researched property. Statistic characteristics for QSAR of PLS (Partial Least Squares) models are satisfactory (R=0,92-0,97; CVR=0,76-0,86). The molecular fragments that increase and decrease antiviral activity were defined and will be demonstrated. This information is useful for design and directed synthesis of novel antiviral agents. Several compounds with predicted high anti-influenza and antiherpetic activities were already synthesized and their activities were confirmed experimentally.

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Influenza infection of the embryonated hen's egg – an alternative model for *in vivo* evaluation of antiviral compounds

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The aim of the present study was to establish an influenza A virus/egg model accompanied by a high mortality rate in 2-weeks-old chick embryos. Further investigations should assess the effect of antiviral drugs on the survival of infected chick embryos. After the preparation of an artificial air chamber into the egg shell, influenza A virus was placed on the chorioallantoic membrane of embryonated hen's eggs. When 1 EID_{50} (50% egg infective dose) of influenza A virus was used nearly 100% of infected chick embryos did not survive the infection up to the day 8 post inoculation. The survival rate of chick embryos could be significantly increased when the antiviral drugs rimantadine, amantadine or zanamivir were administered into the albumen immediately before or after viral inoculation. While rimantadine and amantadine were effective at relatively high doses of 12.5 mg/kg and 25 mg/kg, zanamivir showed a significant antiviral efficacy at concentrations of 2 mg/kg. After pre- and post-treatment with zanamivir, 54% to 58% of the influenza A virus-infected chick embryos survived. The therapeutic effect of amantadine as well as the prophylactic and therapeutic effect of rimantadine was significantly lower. While the survival rate of chick embryos was between 8% and 17% after treatment with rimantadine, between 29% and 33% of embryos survived the infection when amantadine was administered. In conclusion, the chick embryo model described can be used for the reliable *in vivo* evaluation of potential anti-influenza inhibitors. It offers a realistic alternative in comparison to experiments with small laboratory rodents.

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Transdermal delivery efficacy of rimantadine under experimental influenza model in mice.

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Transdermal drugs delivery is a novel pharmacotherapy method for many diseases. The main advantages of this administration method are prolongation of the drug action, absence of the drug concentration hopping, reducing of the adverse reactions risk. This administration method can be concidered for preventive actions and therapy of the virus respiratory infections. This work is aimed at studying of the possibility of transdermal rimantadine delivery for influenza A prophylaxis and treatment in experimental animals. Rimantadine was administrated in the hydrogel matrix (formed from 1.2-propylene glycol and polyvinyl alcohol) in the doses 1 or 2 mg/mouse applied on the shaved mice back 1 day before infection. Experimental and control group mice were infected intranasally with highly pathogenic influen za virus A/PR/8/34 (H1N1). Challenge was carried out using 4 animals for each virus dilution within the range of $1 \cdot 10^{-1}$ to $1 \cdot 10^{-6}$. Death of animals was recorded for 14 days. The results of our investigations had shown high anti-influenza efficacy of rimantadine under transdermal delivery. Difference of LD_{50} between control and experimental groups is 1.75-2.0 $\log_{10}$ when dose of rimantadine was 1 mg/mouse and 2.75-3.0 $\log_{10}$ when drug was administrated in dose 2 mg/mouse. The anti-influenza efficacy of rimantadine after transdermal delivery, has been shown for the first time.

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Isolation of Disoxaril-Dependent Mutant of Coxsackievirus B1

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The antiviral drug disoxaril 5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole selectively inhibits enterovirus replication by binding the hydrophobic pocket in th4e VP1 protein, thus blocking the viral uncoating. In the case of studies of the antiviral activity of disoxaril on Coxsackievirus B1 disoxaril-resistant mutant has been isolated in cell cultures (FL). Three passages of the original Coxsackievirus B1 strain [CoxB1(dis-s/FL)], grown in FL cultures in the presence of 30μM disoxaril resulted in the selection of a drug-resistant mutant [CoxB1 (dis-r30μM/FL)]. Nine consecutive passages of the disoxaril-resistant mutant in FL cell cultures in the presence of 35μM disoxaril resulted in the selection of a disoxaril-dependent Coxsackievirus B1 progeny [CoxB1(dis-d35μM/FL)]. This is the first case of isolation of enterovirus WIN-compound dependent mutant. The one-step growth cycle experimental setup was used for testing the compound effect on the mutants replication.

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Clinical coxsackievirus B3 (CVB3) isolates differ in neutralization pattern, cell tropism, and pleconaril sensitivity

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Twelve clinical CVB3 isolates from patient suffering from encephalitis, meningitis, gastroenteritis, or carditis were compared with the international reference strain Nancy in regard of (1) neutralization pattern; (2) growth characteristics in HeLa cells, Chinese hamster ovary (CHO K1) cells, and human fibroblasts (HuFi); and (3) pleconaril sensitivity. The neutralization titers, obtained with a serum generated against CVB3 Nancy in a rabbit, varied between 1:3000 and 1:18000. Like CVB3 Nancy the clinical virus isolates replicated in HeLa cells inducing a complete cytopathic effect but not at all in CHO K1 cells. Surprisingly, only one of the isolates established a carrier state persistence in HuFi as known for CVB3 Nancy. In the results of the antiviral studies 7 of the 12 clinical isolates were shown to be pleconaril-resistant. The 50 % inhibitory concentration of the pleconaril-sensitive isolates determined in plaque reduction assays was in the range from 5 to 30 ng/ml.

Taken together, these results suggest a broad variability among CVB3 isolates and the necessity of resistance testing for successful therapy.

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Inhibition of Coxsackie B3 Virus Infection *in vitro* and in a Murine Model of Virus-Induced Myocarditis by 2-(3,4-Dichlorophenoxy)-5-Nitrobenzonitrile

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Myocarditis is a common cause of dilated cardiomyopathy, one of the most important single causes of heart transplantation. Coxsackie B viruses are considered to be the principal etiological agents of viral myocarditis, and direct virus-induced damage to the heart has been shown to be the main mechanism underlying myocarditis in the murine model [Horwitz *et al.*, Nature Med. 6: 693-697 (2000)]. We studied whether a broad-spectrum anti-picornavirus agent 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (DNB) [Powers *et al.*, Antimicrob. Agents Chemother. 22: 639-642 (1982)] is able to prevent the development of myocarditis lesions in a murine model. We confirmed that DNB exhibits *in vitro* selective anti-CBV activity. The 50% effective concentration for inhibition of CBV-3induced cytopathic effect in Vero cells and primary human myocard fibroblasts was 1.5 and 8 μg/ml, respectively. The administration of DNB at 250 mg/kg/day subcutaneously at multiple injection sites for a period of 7 consecutive days (starting at 1 day before infection) to four-week-old C3H-mice resulted in a 62% reduction in the number of myocarditis foci in comparison with untreated control animals ($p=1.7 \times 10^{-10}$). This indicates that selective inhibition of the replication of Coxsackie B virus may have a beneficial effect on the development of viral myocarditis. However, early diagnosis of virus-induced myocarditis would be mandatory for an antiviral drug treatment regimen to yield the greatest clinical benefit.

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Effects of Ribavirin on Murine Respiratory Syncytial Virus Disease: Evidence for Immunomodulation

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We have investigated the effects of ribavirin (RBV) in a murine model of RSV disease elcited by intranasal (i.n.) inoculation of BALB/c mice with 0.5 - 2.0 x 10E8 syncytia forming units (sfu) of RSV-A2. RSV disease was characterized by morbidity (ruffled fur, weight loss), mortality, deficits of respiratory function, gross lung consolidation, bronchiolitis, vasculits and interstitial pneumonia. Lung titers of virus ranged from 10E5 - 10E6 sfu/g w.w. lung. RBV administered either i.n. (1 - 10 mg/kg) or subcutaneously (s.c.)(10 - 50 mg/kg) produced a significant reduction of mortality (80 - 100%), deficits of respiratory function (80 - 100%), viral titers (up to 3 logs), consolidation (70 - 100%), bronchiolitis and intersititat pneumonia (60%). Doses higher than 10 mg/kg i.n. and 50 mg.kg s.c. resulted in reduced therapy. Reduction of lung viral titers by RBV was directly proportional to the titer of the infective inoculum and due to an enhanced viral clearance. RBV was therapeutic only when treatment was initiated before 24 hr post-infection. Delaying initiation of therapy beyond 24 hr post-infection had a deleterious effect on disease outcome. Analysis of differential cell counnts in lung sections from RBV treated mice revealed an earlier recruitment of polymorphonuclear neutrophils and lymphocytes in pulmonary infiltrates compared to vehicle treated mice. RBV reduced peak bronchoalveolar lavage fluid levels of TNF-alpha, IL-1beta and IFN-gamma by 33% and IL-12 by 60%. IL-6 levels which remained elevated in vehicle treated mice decreased subsequent to viral clearance by 97% in RBV treated mice. Peak levels of RANTES were reduced (13 - 32%) while MIP-1alpha was unchaged. These observations suggest that the effects of RBV may largely be mediated by an immunomodulatory activity.

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Broad Spectrum Anti-RNA Virus Activities of Titanium or Vanadium Substituted Polyoxotangstates.

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Antiviral activities of 7 polyoxotangstates substituted with titanium (Ti) or vanadium (V) atoms were examined against DFV (Dengue fever virus), FluV-A, RSV, PfluV-2, CDV (canine distemper virus) and HIV-1. Among the 7 polyoxotangstates examined PM-43 [$K5(SiVW_{11}O_{40})$], PM-47 [$K_7(BW_{11}O_{40})$], PM-1001 and 1002 [$K_{10}Na(VO)_3(SbW_9O_{33})_2]26H_2O$ contained V. On the other hand PM-518 [$(Et_2NH_2)_7(PTiW_{10}O_{40})$], PM-520 [$Pri_2NH_2]_5$ [$PTiW_{11}O_{40}$] and PM-523 {[$PriNH_3]_6$ $H[PTi_2W_{10}O_{38}(O_2)_2]\}H_2O$ contained Ti. All compounds showed a broad spectrum antiviral activity against all viruses examined except PM-43, PM-518 and PM-523 which were scarcely inhibitory against PfluV-2, CDV and DFV, respectively. All compounds were inhibitory against HIV-1 replication at an EC_{50} of less than 2.0 μM. The mechanism of anti-HIV action of PM-1001 was displayed that it affected the binding of HIV to cell membrane through the inhibition of gp-120 interactions to cellular receptors. This action and K^+ de_letion activity of PM-1001 from cell surface will be discussed.

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In Vitro Anti-Rhinovirus Activity of a Tetrameric Anti-ICAM-1 Antibody (CFY-196)

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Most human rhinovirus (HRV) serotypes (~90%) share intercellular adhesion molecule-1 (ICAM-1) as an obligate cellular receptor, and previous studies with soluble ICAM-1 and monoclonal antibody to ICAM-1 showed partial protection against HRV colds in humans. We studied the in vitro anti-HRV activity of a tetrameric anti-ICAM-1 antibody (CFY-196) designed by Perlan Therapeutics (San Diego, CA). Three major receptor group HRV serotypes were inhibited by CFY-196 with 50% effective concentrations (EC_{50}s) of 15.9 to 105 ng/ml on two cell lines, HeLa-I and human embryonic lung fibroblast (strain WI-38). No inhibitory effect was observed for one minor serotype, which uses a different cell receptor. Yield reduction assays on HeLa-I monolayers showed that CFY-196 inhibited the replication of HRV 39 in a concentration-dependent manner and, at low viral inocula (32 tissue culture infective doses [$TCID_{50}$]), completely prevented HRV replication when added up to 2 hours after infection at 1μg/ml concentration of CFY-196. It was much more active than a monoclonal antibody to ICAM-1. Single cycle of replication experiments with the addition of CFY-196 at 10μg/ml at different time intervals against high inoculum (2 MOI) showed that the inhibitory effect occurred only when CFY-196 was added before or at the same time as the virus. When viral absorption was carried out at 4°C and subsequent incubation at 34°C, it was found that CFY-196 had to be present during the absorption period to have an inhibitory effect. When CFY-196 at 10 μg/ml was exposed to cells and then removed at different time intervals before HRV-39 inoculation, single cycle studies found that an inhibitory effect was detectable up to 4 hours prior to virus infection. In contrast, a capsid binder (pleconaril) and a 3C protease inhibitor (ruprintrivir) were not inhibitory under such conditions. These results indicate that this tetrameric anti-ICAM-1 antibody inhibits major receptor group HRV serotypes at low concentrations, probably through inhibition of viral absorption, and manifests some residual inhibitory activity in cell culture. This molecule warrants further clinical testing.

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Discovery and SAR studies of a novel class of HCV NS5B RNA-dependent RNA polymerase Inhibitors.

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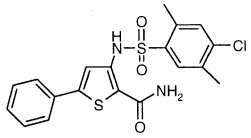
Hepatitis C virus (HCV) infects chronically an estimated 3% of the world's population, often leading to cirrhosis, hepatocellular carcinoma, and failure in later life. Although current therapies, interferon-α, alone or in combination with ribavirin improved the sustained viral suppression rates, it has the disadvantage of frequent and severe side-effects and there is no vaccine available currently. The development of new efficacious and well tolerated low molecular weight agents for treating HCV infection is thus of paramount importance and is currently an intensive area of research. Of several putative viral enzyme targets, the RNA-dependent RNA polymerase has been shown to be critical for viral replication; thus this enzyme represents an attractive target for the discovery of novel antiviral agents. Our screening campaign resulted in the discovery of a novel class of substituted 3-amino thiophene derivatives as potent inhibitor of the HCV polymerase. In this poster we will present enzymatic structure activity relationship studies (SAR) as well as the cell-based replication activities of this class of compounds.



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The Validation of GBV-B as a Surrogate Model for HCV in the Drug Discovery Process

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Previously we have described the development of a GBV-B marmoset model, in which GBV-B replication occurs at consistently high levels in animals and in cultures of primary hepatocytes. We now describe the use of these systems during the lead optimisation phase of the pyrrolidine-5,5-translactam series. This series of compounds are potent inhibitors of HCV NS3 protease. They were also found to inhibit the activity of GBV-B NS3 protease with the same ranking of activity. Three examples of the series were then compared using the HCV replicon system and the GBV-B in vitro replication system. Two compounds (GW3112X and GW0014X), with sub-uM activity in the HCV replicon assay, also showed inhibition of GBV-B replication in culture. A third translacatam (GW0569X) with a 10-fold lower replicon IC50, had little effect on GBV-B replication in culture. Finally, GW0014X was used to treat GBV-B infected marmosets in vivo. Treated animals had more than 3-logs lower virus titre compared to animals given placebo. In fact in 3 of the 4 animals, virus was below the limit of detection. When treatment was removed, 3 of the 4 marmosets remained virus free. In conclusion, we have described a series of HCV protease inhibitors whose activity has been maintained as they have progressed from enzyme assay, into sub-genomic cellular assay, into a full virus replication assay in culture and finally confirmed in the in vivo situation. This demonstrates the utility of the GBV-B model in the drug discovery process.

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Transplantation of HCV-Infected Hepatocellularcarcinoma (HCC) Cell-Line in the HCV-Trimera Mouse System

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The HCV-Trimera mouse is a practical model for HCV infection and is being used for evaluation of potential anti-HCV agents. The model consists of lethally irradiated mice, reconstituted with SCID mouse bone marrow cells, in which human-liver fragments infected ex vivo with HCV had been transplanted. We have adapted this Trimera mouse system for transplantation of HCV-infected HCC cell-line instead of fresh human-liver fragments. HCC cells were infected with high-titer HCV-positive serum from an HCV infected patient, then $1x10^6$ cells were injected into the spleen of a pre-conditioned mouse. These cells populate mice livers as early as 5 days post-transplantation and survive there for approximately two months. Virus loads ((+) strand HCV RNA levels) in mice sera were determined by RT-PCR followed by dot-blot hybridization. Viremia in the HCC cell-line transplanted Trimera mice was detected between days 18 and 50 post transplantation with an infection rate of ~85%, while that in the liver-transplanted mice was detected between days 7 and 30, with a similar infection rate. Anti-HCV activity of an anti-HCV human monoclonal antibody (HCV-Ab^{XTL}68) was observed in Trimera mice transplanted with either human liver tissue or HCC cells. It should be noted that in addition to the possibility for ex vivo infection, the cell-line transplanted mice could also be infected in vivo by i.v. injection of HCV-positive serum two weeks post-transplantation of non-infected cells. Transplantation of HCV-infected HCC cells could extend the time window for evaluation of various anti-HCV agents in the HCV-Trimera model.

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Optimisation of peptide based inhibitors of full length Hepatitis C Virus NS3 protease

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Hepatitis caused by Hepatitis C virus is a serious health problem worldwide. About 2 % of the world population is infected with no etiological treatment available. The bifunctional protease/helicase NS3 protein of HCV is currently a major target for the search of potential anti-HCV drugs. In our work we synthesized and evaluated inhibition of the full length NS3 protease by peptides based on the sequence of the non-prime side of the natural NS3 substrate. Hexa- and pentapeptide inhibitors with three types of residues in the P1 position were designed a) a-carboxylate; b) electrophilic serine trap; c) carboxylic acid bioisosteres. The later group produced the most promising candidates for future improvement: non-thiol containing inhibitors with replacement of C-terminal carboxilate with acyl sulfonamide or a-tetrazole were comparable with thiol-containing ones. Inhibitors from each group were also tested on other serine proteases to confirm selectivity against NS3 protease. We made some efforts to shorten the most active peptides without loosing much of the inhibitory potency. A series of product-based inhibitors spanning the P1-P4 were designed. By varying the groups it was possible to obtain tetra peptides with Ki in low micro molar range. Structure-activity relationships obtained with tetra peptides showed importance of using native, full length NS3 protein for inhibition measurements.

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Synthesis and Evaluation of *S*-acyl-2-thioethyl (SATE) Esters of Modified Nucleoside 5'-Monophospahtes as Inhibitors of Hepatitis C RNA Replication.

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Hepatitis C virus (HCV) infection is a major health problem. Chronic infection can lead liver cirrhosis and hepatocellular carcinoma. An estimated 2-5 % of the world population is infected with HCV. Current approved chemotherapy for the treatment of HCV infection is combination of interferon alpha and ribavirin. Only 40-60% of HCV patients have sustained antiviral response to this treatment, and it is even lower among patients with genotype I HCV. There is a great need for better and novel drugs to fight this infection. Therefore, our research is involved in synthesis and screening of focused class of modified nucleosides to evaluate for inhibition of HCV RNA replication, using enzymatic and cell based (Replicon) assay. We identified a series of modified nucleoside triphosphates as inhibitors of HCV RNA dependent RNA polymerase. Interestingly, we observed that many nucleoside triphosphate that showed good inhibition in enzymatic assay failed to show similar activity cellular assay. This lack of activity we attributed to possible limitation in cell permeation and/or intracellular metabolism to the corresponding triphosphates. To investigate this hypothesis, we synthesized bis *S*-acyl–2-thioethyl esters of modified nucleoside monophosophates. The synthetic strategies to synthesize modified nucleoside monophosphate esters will be presented. These nucleoside prodrugs exhibited several fold improvement in inhibition of HCV RNA replication in cell based replicon assay. The synthesis of labeled SATE prodrugs to study their intracellular metabolism was carried out. The details of metabolism study will be discussed.

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Dissection of the Replicative Cycle of the Bovine Viral Diarrhea Virus to Allow Detailed Time-of-Antiviral Drug Addition Studies

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Bovine viral diarrhea virus (BVDV) is considered to be a valuable surrogate virus for antiviral drug studies concerning hepatitis C virus (HCV). Unravelling the mechanism of action of selective anti-BVDV drugs may yield important insight in possible novel strategies to inhibit the replication of HCV or other viruses that belong to the Flaviviridae Family. We here present a study in which we dissected the replication cycle of BVDV with the aim of carrying out detailed time-of-drug addition studies. Time-of-drug addition studies should ideally be carried out during one single replication cycle of the virus. Therefore, real-time quantitative RT-PCR was employed to define the precise length of the replication cycle of BVDV (strain NADL). New progeny virus [containing positive (+) stranded RNA] was first detected in the supernatant of infected MDBK cell cultures at about 12 hr post infection. Intracellularly, newly produced viral (+) stranded RNA, as well as (-) stranded RNA, was detected first at about 6 hr post infection. In addition to quantifying the appearance of intracellular and extracellular RNA, the production of viral antigens [gp48, gp53, gp62 and p80-p125] by means of Western blotting was monitored as an additional parameter of the replication cycle of the virus. Next, it was determined how long after infection, and at which time relative to the appearance of viral RNA and antigens in the untreated cultures, the addition of 3-[((2-dipropylamino) ethyl) thio]-5H-1, 2,4-triazino[5,6-b]indole, a known inhibitor of the BVDV RNA-dependent RNA polymerase [Baginski *et al.,* Proc. Natl. Acad. Sci. USA 97:7981-7986 (2000)] could be delayed before the antiviral activity was lost. The method presented should be useful in acquiring initial information on the molecular target of selective inhibitors of BVDV replication.

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Synergistic Direct Interactions between IFN and RBV against both BVDV and YFV as Surrogate Models of HCV Replication

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The current treatment for hepatitis C virus (HCV) is a combination therapy of interferon-α (IFN) and ribavirin (RBV). Monotherapy of HCV infection with RBV does not reduce viral loads nor does it lead to a sustained virologic response (SVR). IFN monotherapy can reduce viral loads and leads to SVR in ~20% of patients. The combination therapy of IFN and RBV is much more effective clinically than either drug alone, leading to ~40% SVR. IFN and RBV are thought to act as antiviral agents through both direct and indirect mechanisms, however the contribution of these activities to the observed clinical synergy is not known. We used MacSynergy II to analyze the effects of combining these drugs *in vitro* on antiviral activity and drug cytotoxicity. We observed statistically significant synergistic drug-drug interactions between the antiviral activity of IFN and RBV at physiologically relevant concentrations of the drugs against both Bovine viral diarrhea virus (BVDV) and yellow fever virus (YFV) used as surrogate models of HCV replication. These results suggest that the synergy of antiviral activity of IFN and RBV can be accounted for by the direct effects of the drugs on cells.

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Potent Inhibition of NTPases/Helicases of the West Nile Virus (WNV) and Other Flaviviridae by Ring-Expanded ("Fat") Nucleoside and Nucleotide Analogues

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A series of ring-expanded ("fat") nucleoside analogues (RENs) containing 6-aminoimidazo[4,5-*e*][1,3]diazepine-4,8-dione ring system have been synthesized as potential inhibitors of NTPases/helicases of *Flaviviridae*, including the West Nile Virus (WNV), Hepatitis C Virus (HCV), and Japanese Encephalitis Virus (JEV). In order to assess the selectivity of RENs against the viral enzymes, a truncated form of human enzyme $Suv3_{(\Delta 1\text{-}159)}$ was also included in the study where necessary. As part of the exploration of structure-activity relationships (SAR) of the target ring system, the synthetic endeavor largely focused on structural variations at position 1 and 6 of the heterocyclic base. The structural modifications at position 1 included changes in both the type of sugar moieties (ribo- and 2'-deoxyribosugars), and the mode of attachment (α *versus* β anomeric configuration) of those sugars to the heterocyclic base. The modifications at position 6 included changes in different alkyl, aralkyl, and aromatic substituents attached to the amino group. The target RENs were biochemically screened separately against the helicase and ATPase activities of the viral NTPases/helicases. A number of RENs, which included at least one from each category (ribo-, deoxyribo-, aglycons, α-, and β–anomers), inhibited the viral helicase activity with IC_{50} values that ranged in micromolar and submicromolar concentrations, and exhibited selectivity between the viral and human enzymes.

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Ribavirin efficacy against West Nile virus after multiple passages in cell culture. C. W. Day, J.D. Morrey, R.W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, Utah, USA.

Nucleoside analogues have been reported to cause error-prone replication in RNA viruses and viral extinction after multiple passages. Ribavirin was evaluated for similar effects against West Nile virus. Multiple cell lines were shown to support replication of West Nile virus, including CV-1, MRC-5, C127i, L929, Vero, MA104, BHK, A549 and HeLa. Ribavirin was shown to have a sustained effect against low inocula (5 CCID-50) of West Nile virus in CV-1 and MA-104 cell cultures, but this may be largely due to metabolic inhibition to the cell rather than antiviral specific effects. Subtoxic doses of ribavirin (≤100 μg/mL) were evaluated for antiviral effect against higher inocula of West Nile virus in various cell lines. The virus was diluted and passed multiple times to fresh cells to determine whether cumulative mutations would lead to viral extinction. Direct virus counts were measured using Vero cells. The virus produced $>10^8$ $CCID_{50}$ per mL in L929 cells consistently over 28 passages, and continued to thrive after many passages in other cell lines. While ribavirin may cause error-prone replication, it may not affect the infectivity of the West Nile virus at subtoxic doses.

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A Neutral Red Uptake Assay for the Rapid Screening of Antiviral Compounds Against the Filoviruses, Ebola and Marburg

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The filoviruses, Ebola and Marburg, members of the family *Filoviridae*, are known to cause sporadic epidemics of viral hemorrhagic fever among human and nonhuman primates. Due to the high mortality and morbidity caused by these viruses, there is increasing concern that one or both of these viruses may be used as agents of bioterrorism and/or biowarfare. Currently there are no available antiviral therapies for filovirus infections; and therefore, there is an urgent need to identify lead compounds that can be developed as effective therapies in the event of future outbreaks. Therefore, we developed an in vitro neutral red uptake assay for rapidly screening potential antiviral compounds against Ebola and Marburg viruses. To identify any differences in drug sensitivities we tested the Ebola strains: Zaire'76, Zaire '76 mouse-adapted, Zaire'95, Reston, Sudan, and Ivory Coast, and the Marburg strains: Musoke and Ravn. We used this assay to evaluate the antiviral activities of a number of potential antiviral compounds. To validate the assay, we screened multiple adenosine analogues, most notably, the S-adenosyl-L-homocysteine hydrolase inhibitors, carbocyclic 3-deazaadenosine and 3-deazaneplanocin A, which have been shown previously to inhibit the replication of Ebola virus in vitro and in a mouse model of Ebola infection. The nucleoside analogue, ribavirin, which has antiviral activity against a broad range of RNA viruses, showed no activity against filovirus replication. This simple and rapid in vitro neutral red uptake assay will significantly expedite the screening of antiviral compounds for activity against these important public health and potential bioterrorism/biowarfare agents.

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Human Miniantibodies Specific to Ebola virus

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A collection of human scFv phage antibodies against Ebola, Zair, virus have been obtained form a combinatorial phage library of human single-chain antibody fragments, scFv, (Medical Research Council Centre, Cambridge, England). Affinity and crossreactivity of the selected antibodies in binding reactions with active and inactive Ebola and Marburg viruses have been assayed by indirect ELISA. In addition, target filovirus proteins for the selected antibodies have been determined using immuno-blot analysis. Capability of the selected antibodies to neutralize Ebola virus infectivity was estimated in plaque-reduction neutralization test using eukaryotic cells monolayer.

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Antiviral activity of new thiosemicarbazone derivatives. C.C. García*, B.N. Brousse**, M.J. Carlucci*, A.G. Moglioni**, M.M. Alho***, G.Y. Moltrasio**, N.B. D'Accorso***, E.B. Damonte. *Laboratorio de Virología, Dpto. de Química Biológica, FCEN, **Dpto. de Química Orgánica, FFyB, ***Dpto. de Química Orgánica, FCEN, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina.

A considerable number of thiosemicarbazone (TSC) derivatives has been reported as antibacterial, antiviral and antiproliferative compounds. In particular, certain TSCs showed a selective inhibition of herpes simplex virus and human immunodeficiency virus infections. In this study, we report the inhibitory activity against Junin virus (JUNV), an RNA virus member of the family Arenaviridae and agent of Argentine hemorrhagic fever (AHF), of several TSCs, synthesized from aromatic ketones and terpenones, and the corresponding thiadiazoline (TDZ) derivatives, obtained by heterocyclization. The toxicity for Vero cells of 25 compounds, including TSCs, TDZs, unprotected TDZs and intermediate products, was first investigated by MTT method. According to compound solubility, the highest concentration assayed was 200 μM and the values of CC_{50} varied in the range 36.1 - >200 μM. Antiviral activity was evaluated by a virus yield inhibition assay in Vero cells. Only three TSC derivatives were totally inactive against JUNV and most of the remaining TSCs inhibited JUNV replication with EC_{50} values in the range 3.4-100 μM. The most active inhibitors showed similar or higher efficacy in vitro than ribavirin, the only drug known to be of any benefit in the treatment of patients with arenavirus infection. By contrast most of the ten TDZs assayed were not active against JUNV. No conclusive structure-activity relationships could be established but systematically higher activity was associated to TSCs synthesized from aromatic ketones. The active compound **4a**, TSC of tetralone, was chosen for further characterization of the mode of action of this kind of compounds. No direct virucidal effect on JUNV virions was shown by **4a**. Time course experiments indicated that the inhibitory action was exerted on a late step of JUNV replicative cycle. In addition, no alterations in viral protein synthesis in **4a**-treated cells were observed suggesting that the final stage of virion maturation is the target of the compound in JUNV infected cells.

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Construction of Pseudorabies virus (PRV) virion harboring Fc region of mouse IgG on its envelope.

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We established RK13 cell expressing a chimeric antibody-like molecule, containing Fc region of mouse IgG1. The chimeric protein was a type II membrane protein in such an orientation that Fc portion projected away from the cell surface. The cell line expressing the chimeric protein was designated as RK/Fc. When RK13 or RK/Fc cells were cultured with mouse macrophages, RK/Fc cells were damaged much more than RK/13 cells, indicating that the Fc region can be recognized by Fc receptors of macrophages. The activation of the complement pathway by the molecule was not detected. PRV propagated in RK13 or RK/Fc cells were neutralized by rabbit complement in the presence of antibody recognizing PRV antigen. In the presence of anti-mouse IgG antibodies, only the PRV propagated in RK/Fc cells were neutralized. In addition, the Fc was detected from the purified virion by ELISA. These data indicate that the cell derived Fc is incorporated PRV virion. The PRV virion harboring the chimeric Fc has a potential for killing vaccine, which enhance the uptake by antigen presenting cells via FcRs.

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Cell-based screening for antivirals against negative-strand viruses

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Effective antiviral therapy is lacking for most human viral pathogens including negative-strand RNA viruses such as respiratory syncytial virus (RSV). Apath has developed a screening platform based on two cell-based assays that facilitate the discovery of antivirals against RSV and other negative-strand RNA viruses. Both assays are based on expression of a reporter gene from a viral minigenome. The primary screening assay is independent of virus infection. Baby hamster kidney (BHK-21) cells that express T7 RNA polymerase are transfected with five plasmids: an RSV minigenome containing *lacZ* and four T7-expression plasmids for the viral proteins (N, P, L and M2-1) required for RSV replication and transcription. These indicator cells are produced on a large scale and frozen in aliquots convenient for screening in 96-well plates. Using a chemiluminescent β-galactosidease (β-gal) assay we routinely obtain a signal of up to 10^6 LCPS/well at 24 hours with a signal-to-noise ratio around 1000. The assay exhibits good reliability with a coefficient of variation (CV) of 8-14%. To validate the use of the assay for screening, we tested the effect of ribavirin on β-gal expression. The IC50 of ribavirin in this assay is 4-12 uM which is within the range observed using other methods such as the plaque reduction or CPE inhibition assays. Our other assay is essentially a virus yield assay whereby RSV is titered by inoculation onto cells transfected with the *lacZ* minigenome. β-gal expression is proportional to the RSV titer and the IC50 for ribavirin in this assay is very similar to what we find in the infection-independent assay. We have developed a screening algorithm in which we use the infection-independent assay to screen libraries of compounds and then putative inhibitors are confirmed in the viral yield assay. We are currently applying this platform to other negative-strand RNA viruses for which reverse genetic systems are available. This is particularly attractive approach for BL3 or BL4 agents (e.g. filoviruses) since the cell-based screening is performed without infectious virus.

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The potentiation of interferon-α and –β antiviral activities with hydroxocobalamin against vesticular stomatitis virus (VSV), herpes simplex virus type 2 (HSV-2), and human hepatitis B virus (HBV) Yunhao Gong[a], Aleksandra Pastrak[b], Izabelle Gadawski[a], Dorothy Cheung[a], Teresa Tam[a], Tony Cruz[b], Stephen Sacks[a,c]
a. Viridae Clinical Sciences Inc., 1134 Burrard Street, Vancouver, British Columbia, V6Z 1Y8; b. Transition Therapeutics, Inc. 415 Yonge St., Suite 1103, Toronto, Ontario, M5B 2E7; c. Department of Pharmacology and Therapeutics, The University of British Columbia, 2176 Health Sciences Mall, Vancouver, British Columbia, V6T 1Z3, Canada

The potentiation of interferon (IFN) anti-viral activity with hydroxocobalamin (HC), on VSV was tested using MRC-5 cells by CPE (cytopathic effect) inhibition assays. Strong potentiation of an anti-viral effect was observed for HC (1-1000 μg/ml) on IFN-β (5-500 IU/ml) activity against VSV at all concentrations tested and on IFN-α activity when the concentrations of HC and IFN-α were higher than 100 μg/ml and 50 IU/ml, respectively. Based on the outcome of the combination index analysis, the anti-viral effects were synergistic.

The potentiation of IFN activity with HC on HSV-2 was tested in a similar way to VSV. Strong synergism was observed for HC on IFN-α activity against HSV-2 when the concentrations of HC and IFN-α were higher than 10 μg/ml and 10 IU/ml, respectively. Moderate to strong potentiating effects were also observed for HC on IFN-β activity against HSV-2.

The potentiation of IFN anti-viral activity with HC on HBV was tested on HepG2.2.15 cells. Moderate to strong synergistic effect was shown for HC on IFN-β activity against HBV at the concentrations higher than 10 μg/ml and 100 IU/ml, respectively. Moderate synergistic response was observed for HC on IFN-α activity against HBV when the concentrations of HC and IFN-α were higher than 100 μg/ml and 500 IU/ml, respectively.

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Discovery of the First Inhibitors of the Cooperative Binding of Human Papillomavirus Type 11 E1 and E2 Proteins. C. Yoakim[1]; P.W. White[2] , W.W. Ogilvie[1]; N. Goudreau[1] ; G.A. McGibbon[1]; J.A. O'Meara[1]; J. Naud[1], B. Haché[1], L. Doyon[2]; M.G. Cordingley[2]; J. Archambault[2]. (1) Dept. of Chemistry and (2) Dept. of Biological Sciences, Boehringer Ingelheim (Canada) Ltd., Laval, Quebec, Canada.

Human papillomaviruses (HPV) infect the basal cells of the epithelium and are host and tissue-specific. HPV are small, non-enveloped viruses containing a double stranded covalently closed circular DNA genome (approximately 8 kb), which encodes 6 well characterized early and 2 late gene products. Only two virally encoded proteins, E1 and E2, are required for replication of the genome. We have discovered a series of inhibitors of the cooperative binding of HPV11 E1 and E2 proteins to the viral origin of replication using high throughput screening. These inhibitors feature an indandione system spiro-fused onto a substituted tetrahydrofuran ring. The structure elucidation of the active species and biochemical characterization of these inhibitors will be presented.

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Collaboration with the National Cancer Institute (NCI) in the Development of Antiviral Therapeutics.

Bjarne Gabrielsen, Senior Advisor, Drug Discovery and Development, Technology Transfer Branch, NCI-Frederick, Frederick, MD 21701

NCI scientists have historically played a role in the discovery and development of anti-viral drugs in addition to their mission of developing antitumor therapeutic agents. Such discoveries have resulted from collaborations involving NCI scientists and/or the use of unique NCI resources such as the DTP-Natural Products Repository. This talk will: a)present an overview of several recent NCI discoveries focused on the area of antiviral therapeutics; b) discuss modes of collaboration with the NCI; and, c) highlight new opportunities for collaboration and utilization of unique NCI resources.

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Identification of Hepatitis C virus NS5B RNA-dependent RNA polymerase inhibitors from high throughput screening assay

M.K.LEE, H.S.KIM, J.-R.CHOI, Y.Z.KIM, W.Y.CHO, Y.G. CHO, G.W. KIM, T.G.KIM, J.-T. HWANG, D.G. CHO, K.Y. ROH, H.S. YOON, H.S.LEE, H.J.KIM and J.KIM
LG Life Sciences, R&D Park, Daejeon, Republic of Korea

The nonstructural protein 5B (NS5B) of hepatitis C virus (HCV) is the viral RNA-dependent RNA polymerase (RdRp), the essential catalytic enzyme for the viral replication. We cloned from Korean type HCV patient serum, expressed and purified the soluble recombinant HCV NS5B lacking the C-terminal 24 amino acids residues. With the highly purified NS5B protein, we established in vitro systems for RdRp activities to identify potential polymerase inhibitors. The Bisaryl, rhodanine, barbituric acid, pyrimidone family compounds were found to be potent, highly specific inhibitors of NS5B from high throughput screening assay, applied to the scintillation proximity assay (SPA) systems and validated through filter-binding assay and gel-based RdRp assay. Furthermore, we also investigated the ability of these compounds to inhibit NS5B-directed viral RNA replication using the Huh7 cell-based HCV replicon system. The investigation is potentially very useful for the utility of such compounds as anti-hepatitic agent.

Oral Session VII: Respiratory Viruses, Late Breaker Presentations

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Inhibition of Human Rhinovirus (HRV) Infection by a Tetravalent Anti- ICAM-1 Fab Fusion Protein, CFY196

C. H. Charles, G. X. Luo, L. A. Kohlstaedt, L. Cao, J. H. Williams, G. Husted, A. Phillips, I. G. Morantte, E. Gorfain, A. Miller, O. Turan and F. Fang. *Perlan Therapeutics, 6310 Nancy Ridge Drive, Suite 102, San Diego, CA92121*

HRV, the major cause of the common cold, binds to host receptor ICAM-1 to infect. Prior works on HRV receptor-blocking monoclonal antibody reveal that insufficient avidity accounts for unsatisfactory efficacy of an antibody. To solve this problem, we have developed a proprietary Multi-Valence™ platform technology for improving avidity by making multivalent recombinant antibodies and proteins. We have designed tripartite proteins consisting of a Fab molecule fused with a hinge derived from human IgD, further linked to polymerization domains derived from human coiled-coil proteins. Using the technology, we have generated a tetravalent humanized Fab fusion protein against ICAM-1, CFY196. Comparing to bivalent monoclonal antibodies, CFY196 shows over 100 times affinity improvement due to decreased rate of dissociation (k_{off}). The kinetic superiority of CFY196 has led to remarkable improvements in the ability to prevent and interrupt HRV infections in vitro. CFY196 has superb stability, is produced in bacteria and purified as soluble pre-assembled homogeneous tetrameric complex at high yield. CFY196 is a promising molecule for prophylaxis and therapy of the common cold. The Multi-Valence™ platform technology is also adaptable for other applications where increased avidity is desirable.

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Inhibition of Respiratory Syncytial Virus Fusion by the Small Molecule, VP-14637 via Specific Interactions with the F Protein.

J. L. Douglas, M. L. Panis, E. Ho, K.-Y. Lin, S. H. Krawczyk, D. M. Grant, R. Cai, S. Swaminathan, and T. Cihlar. Gilead Sciences, Foster City, CA

Human respiratory syncytial virus (RSV) is a major cause of respiratory tract infections worldwide. Several novel small-molecule inhibitors of RSV have been identified, but they are still in pre-clinical or early clinical evaluation. One such inhibitor is a recently discovered triphenol-based molecule VP-14637 (ViroPharma). Initial experiments indicated that VP-14637 acts early in the virus life cycle, possibly by inhibiting viral attachment or fusion. Here we present data that demonstrates VP-14637 does not inhibit RSV binding, but inhibits fusion through interactions with the viral fusion protein, F. RSV variants with >1000-fold resistance to VP-14637 were selected that contained mutations located in two distinct regions of the F protein, the heptad repeat 2 (HR2; mutation F488Y) and the intervening domain between heptad repeat 1 (HR1) and HR2 (mutation T400A). No mutations arose in HR1, as might be expected for an inhibitor that directly disrupts the heptad repeat interaction, suggesting that VP-14637 might have an alternative mechanism of action. Interaction studies using [^{3}H]VP-14637 revealed a specific binding of the compound to RSV-infected cells (Kd ~ 5nM). Further analysis using a transient T7 vaccinia expression system indicated that the RSV F protein is sufficient for this interaction. Interestingly, vaccinia-expressed F proteins containing the resistance mutations exhibited greatly reduced binding to VP-14637. Despite segregation with the membrane fraction following incubation with intact RSV-infected cells, the compound did not bind to isolated membranes from RSV-infected cells. In addition, no significant binding of VP-14637 was detected at temperatures ≤ 22 °C. Therefore, we propose that VP-14637 inhibits RSV through a novel mechanism involving an interaction between the compound and a transient conformation of the RSV F protein.

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Efficacy and Tolerability of Long-term Pleconaril Chemoprophylaxis for Picornavirus Illness in Adults.
FG Hayden, S Liu, SA Villano, and M McKinlay, University of Virginia, Charlottesville, VA, USA and ViroPharma Inc., Exton, PA, USA

The orally administered, capsid-binding, anti-picornavirus drug pleconaril has shown to be effective for treatment of picornavirus colds in adults. We conducted a randomized, double-blind, placebo (P)-controlled study at 13 centers to assess the tolerability and efficacy of pleconaril in preventing picornavirus morbidity. During August-October 2001, 1,069 healthy adults were enrolled at 13 centers and randomized in a 1:1:1 ratio to pleconaril 400 mg QD or BID or P for 6 weeks. The main efficacy outcome was laboratory proven, self-diagnosed picornavirus colds, which were observed in 20% of P, 13% of pleconaril QD, and 12% of pleconaril BID recipients ($P \leq 0.01$, pleconaril QD or BID vs P). Picornavirus-associated missed school or work (32-36% reductions), functional impairment (38-43%) and sleep disturbance (38-43%) were all significantly reduced in pleconaril recipients compared to P. Discontinuations due to possible adverse events were observed in 3% P, 4% pleconaril QD, and 6% pleconaril BID. Headache, nausea, and diarrhea were among the most frequently reported adverse events in all treatment groups. Among women taking oral contraceptives, menstrual disorders were reported 2- to 3-times more frequently in the pleconaril groups compared to P. Pleconaril is the first oral antiviral chemoprophylactic agent proven to reduce picornavirus morbidity in adults. Pleconaril was generally well tolerated when used for prolonged dosing, although it showed evidence of drug interactions with oral contraceptives.

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Arenavirus inactivation with conservation of virion surface glycoproteins and blockade in viral transcription.
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Arenaviruses contain bisegmented single-stranded RNA and express five proteins. One of them, the Z protein, presents a conserved RING finger motif that binds Zn and makes this protein an attractive target for antiviral therapy. In previous studies it was reported the effective inhibitory action against the arenaviruses Junin (JUNV) and Tacaribe (TCRV) of two types of antiretroviral Zn finger compounds provided by the National Cancer Institute (USA), the intermolecular aromatic disulfide NSC20625 and the dithianes NSC624151 and 624152. These compounds were able to inactivate virions by direct contact as well as to reduce virus yields from infected Vero cells. In this report, we demonstrate that treatment with these compounds rendered non-infectious virus particles but preserving the conformational integrity of the virion envelope glycoproteins GP1 and GP2. This conclusion is supported by the following results: 1) binding of inactivated virions to cellular receptor was performed with the same efficacy as native virions; 2) internalization via the fusion between viral and endosome membranes was not affected in inactivated virions; 3) after inoculation of adult mice with 10^4 PFU of either control or NSC20625 treated JUNV or TCRV, comparable levels of antibodies were induced indicating that the immunogenicity of viral glycoproteins was preserved; 4) no alterations in viral glycoproteins of purified virions were detected by SDS-PAGE. To further understand the antiviral mechanism, the ability of inactivated JUNV virions to perform genome transcription and replication on Vero cells was studied. No amplification products were detected by RT-PCR with specific primers corresponding to genomic and antigenomic NP (nucleocapsid protein) and GPC (glycoprotein precursor) sequences. Thus, inactivation of arenaviruses with these compounds lead to a blockade in viral transcription, probably due to an interaction with the RING finger motif of the viral Z protein.

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LB80380, a Novel Nucleoside Phosphonate, is a Very Potent Anti-HBV Agent.

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LG Life Sciences, R&D Park, Daejeon, Republic of Korea

We have designed and synthesized many novel phosphonate nucleotides, with a cyclopropyl group in their acyclic backbones, that exhibit a unique conformation to result in potent and selective anti-HBV activities. LB80380 and its parent molecule, LB80317, were effective in vitro against wild type and lamivudine-resistant HBV with EC50 of 0.5-10 uM. LB80380 was effective at doses ranging from 0.2 to 100 mg/kg in transgenic mice expressing HBV when treated orally once a day. Woodchucks were treated orally for 28 days at doses of 5 & 15 mg/kg/day of LB80380. WHV DNA viral load was reduced by greater than six logs after four weeks. Mitochondrial toxicity and kidney toxicity were shown to be very low in in vitro models. Also LB80380 has shown excellent safety and pharmacokinetics profiles. Oral bioavailabilities of LB80380 are 20% to 60%, which varies according to animal species. The Cmax and AUC were significantly greater than those estimated for an effective concentration (EC50) of LB80380, and the EC50 levels were maintained until 24 hours. LB80380 is in human clinical phase II trial for the treatment of chronic human HBV infections. The details of synthesis, efficacy, safety and pharmacokinetics of LB80380 will be presented.

First Author Index – Abstract Number

Seventeenth International Conference on Antiviral Research

Tuscon, Ariz., USA

May 2–7, 2004

Dear Friends and Colleagues,

The 17th International Conference on Antiviral Research will be held in Tucson, Arizona, USA. The conference will begin on May 2, 2004 (Sunday) and will end at 1 pm on May 6, 2004 (Thursday). All scientific sessions will be held at the Hilton Tucson El Conquistador Resort. There will be a Welcome Reception on Monday evening, May 3, and a Conference Banquet Wednesday evening, May 5, for all registered attendees.

The purpose of the Conference is to provide an interdisciplinary forum at which investigators involved in basic, applied, and clinical research worldwide can meet to review recent developments in all areas of antiviral research. Conference participants will hear from leading “overview” speakers on the most current related topic. A Number of oral and poster presentations will be selected from submitted abstracts.

Tucson is Arizona’s second largest city and is home to more than 800,000 people. The city's geography is a postcard image of cactus forests, rolling hills, and stony mountains. Tucson lies in the borderland, a region that blends the cultures of the United States and Mexico, and has a history of settlement by ancient Native American peoples, Spanish explorers, and frontiersmen.

Tucson was formally founded in 1775, about the time the United States signed the Declaration of Independence. Locally, the city is still called the Old Pueblo for the adobe fortress or "presidio" that marked its early borders. Over the past three centuries, Tucson has grown from Native American farming community, to Spanish outpost, to dusty frontier town to the mature Southwestern metropolis seen today.

For a perfect introduction to Tucson and Southern Arizona's past, visitors can take a self-guided Downtown Walking Tour and stroll through a restored 19th century neighborhood and the site of the original Spanish presidio. To learn about Tucson's significant aviation history, be sure to visit Pima Air & Space Museum, the world's largest privately-funded air museum. For those interested in native wildlife, The Arizona-Sonora Desert Museum is one of the most notable wildlife parks in the world. Just south of Tucson lies the Patagonia-Sonoita Creek Preserve, a pristine wildlife sanctuary.

There are more than 3,000 eateries in Tucson, including many award winning restaurants including authentic Mexican cuisine, form Sonoran to Mexico City style as well as southwestern cuisine and Wild West steakhouses. Visit the natural wonders of the desert on horseback or on foot. You may also see the old West in action at Tucson Studios with a full array of live shows.

Tucson enjoys more sunshine than any other city in the United States, about 350 days each year. The days, with very few exceptions, are warm and sunny. The mild temperatures during spring, fall, and winter are legendary. Temperatures rise in the summer, but low humidity helps even the warmest days feel comfortable.

We hope you will take advantage of this opportunity to combine an important learning experience with a wonderful travel experience to the Western United States and join us in Tucson, Arizona for the 17th International Conference on Antiviral Research.

ISAR Conference Committee

Hilton El Conquistador Resort
Tucson, Arizona
May 2 – May 6, 2004

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Additional Information:

Information on housing and travel arrangements, the scientific program, instructions for submitting abstracts, hotel accommodations and conference registration forms will be included in the second announcement, which will be sent to all members of ISAR. The information will also be available after July 1, 2003 on our web page – www.isar-icar.com

If you are not a member and wish to receive this information, please see our web page or contact the Organizing Secretariat.

Future Conferences

2004 – May 2-7, Tucson, Arizona, USA

2005 – April-May, Barcelona, Spain

2006 – San Juan Puerto Rico - Tentative