Oral Session I: Retrovirus Infections I

1

Anti-HIV and HBV activity of 2'-Fluoro-2',3'-unsaturated *L*-Nucleoside (*L*-d4N) Analogs

K. Lee,[+] Y.S. Choi,[+] E.A. Gullen,[¥] Y.C. Cheng,[¥] R.F. Schinazi[#] and C. K. Chu [+*]
[+]College of Pharmacy, The University of Georgia, Athens, GA 30602
[¥]Yale University School of Medicine, New Haven, CT 06520 and [#]Emory University School of Medicine/VA Medical Center, Atlanta, GA 30033.

L-Nucleosides, such as 3TC, FTC, L-FMAU have emerged as promising antiviral agents with favorable toxicity profiles. Recently, *L*-d4C and *L*-Fd4C have been identified as potent antiviral agents against HIV and HBV. The synthesis of *L*-d4-purine nucleosides were also reported, among which *L*-d4A exhibited significant anti-HIV and anti-HBV activities. However, d4N is unstable under acidic conditions, resulting in a glycosyl bond cleavage, which limits the oral bioavailibility. As a means of circumventing this shortcoming, we introduced a vinylic fluoride moiety into *L*-nucleosides.

Our synthethic strategy was based on direct coupling of sugar moiety with a variety of heterocycles to obtain our target compounds. It was envisioned that 2,3-unsaturated sugar moiety bearing fluorine atom at 2'-position is amenable under condensation reaction conditions in the presence of Lewis acid. Acetate sugar moiety was readily prepared from 1,2-*O*-isopropylidene-*L*-glycealdehyde *via a* (*R*)-2-fluoro-butenolide intermediate. The key intermediate was successfully condensed with a variety of hetero-cycles to give the target compounds.

The newly synthesized compounds were evaluated for their antiviral activity against HIV-1 in human PBM cells and HBV in 2.2.15 cells. Preliminary results indicated that cytosine, 5-fluorocytosine, and adenine derivatives demonstrated significant activity against HIV (EC_{50} 0.51, 0.46, and 1.5 μM, respectively) and HBV (EC_{50} 0.35, 0.6, and 0.5 μM, respectively) with cytotoxicity greater than 100 μM in human PBM, Vero, CEM and HepG2 cells.

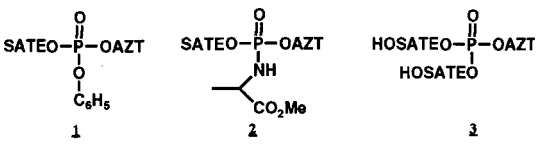
In view of the potent anti-HIV and anti-HBV activity, the lack of toxicity and increased chemical stability, *L*-2'-F-d4N warrants further investigation as potential antiviral agents (This research was supported by NIH grants AI32351 and AI33655 and the Department of Veterans Affairs).

2

Synthesis and antiviral evaluation of new pronucleotide series

J.-L. Imbach, C. Périgaud, Gilles Gosselin
Laboratoire de Chimie Bio-Organique, UMR CNRS-UM II
Case Courrier 008, Université Montpellier II
Place Eugène-Bataillon, 34095 Montpellier Cedex 5 (France)

We will comment in this presentation on the rational design of some pronucleotide (mononucleotide prodrug) series in relation with their expected decomposition and with their pharmacokinetic parameters. The synthesis of monoSATE prononucleotide series such as 1 and 2 will be described for the first time.



1 2 3

Decomposition mechanism of 1 and 2 in total cell extracts will be presented and the structure of their metabolites determined by HPLC/MS coupling. Both series deliver the 5'-mononucleotide. In addition, in order to increase the water solubility of bis(*t*BuSATE) pronucleotides, the bis(hydroxy-*t*BuSATE) series 3 will be introduced.

Comparative stability data and Log P values of pronucleotides 1-3 will be presented and the scope and limitations of the pronucleotide approach will be discussed.

3

HIV infection of human lymphoid tissue *ex vivo*: Differential pathogenesis of CCR5- and CXCR4-tropic HIV-1 isolates.

L. Margolis, J.-C. Grivel, S. Glushakova, M. Penn*, M. Goldsmith*
National Institute of Child Health and Human Development, Bethesda MD 20892 USA and *Gladstone Institute of Virology, San Francisco, USA.

Human lympoid tissue cultured *ex vivo* preserves its cytoarchitecture, mounts a secondary humoral immune response and supports productive HIV-1 infection without exogeneous activation, thus providing a new system to study HIV pathogenesis. HIV infection results in an isolate-dependent CD4+ T cell depletion, simulating a hallmark of HIV disease *in vivo*. CCR5-tropic (R5) HIV-1, predominant during primary infection deplete CD4+ T cells mildly whereas CXCR4-tropic (X4) or R5X4 viruses, that *in vivo* often emerge in late stages of HIV disease, deplete CD4+ T cells severely. Infection with X4, but not R5 viral isolates prevents the development of immune response in *ex vivo* tissues indicating on the causative relationship between virus tropism and its cytopathicity. To establish viral and cellular mechanism of the differential pathogenesis of R5 and X4 HIV-1 isolates, we inoculated explants of human tonsillar tissue *ex vivo* with paired chimeric viruses that are genetically identical (isogenic) except for select envelope determinants specifying reciprocal tropism for CXCR4 or CCR5. X4 HIV-1 massively depleted CD4+ lymphocytes while matched R5 viruses depleted such cells only mildly despite comparable viral replication kinetics. We showed that the ranges of specific targets within the T cell subsets for X4 and R5 isolates are different: R5 HIV-1 isolates deplete only CCR5+ expressing CD4+ T cells which constitute a minority (<10%) of CD4+ T cells, whereas X4 isolates deplete CD4+ T cells of all subsets, leading to distinct consequences for the lymphocyte population overall. These findings demonstrate that HIV-1 co-receptor utilization determined by envelope sequences is a causal factor in CD4+ T cell depletion in human lymphoid tissue. The differential cytopathic effects of R5 and X4 HIV-1 isolates in lymphoid tissue are caused by difference in the spectrum of R5 and X4 targets among CD4+ T lymphocytes. Possible clinical correlates of these results, and the potential of the aove described system for studying the effects of anti-virals on HIV-1 replication and CD4+ T cell depletion in the context of human lymphoid tissue will be discussed.

4

Blockade of CXCR4 Prevents the Emergence of X4, T-Cell Tropic, Syncytium-Inducing Strains of HIV-1

J.A. ESTÉ[1], C. CABRERA[1], A. GUTIERREZ[1], J. BLANCO[1], D. SCHOLS[2], B. CLOTET[1], G. HENSON[3], G. BRIDGER[3], and E. DE CLERCQ[2]
[1]Institut de la Recerca de la SIDA-Caixa, Hospital Universitari Trias i Pujol, Badalona, Spain. [2]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium. [3]AnorMED Inc. Langley, BC, Canada.

X4 strains of HIV-1 are T-cell tropic, rapidly replicating, syncytium-inducing (SI) strains that use the CXCR4 receptor as entry cofactor in $CD4^+$ cells. Emergence of X4 strains is associated with the rapid decline of $CD4^+$ T cells that heralds the onset of AIDS. Conversely, macrophage-tropic, non-syncytium inducing (NSI) strains (R5 strains) that use CCR5 are commonly transmitted during primary infection. The X4 strains may not be (detectably) present upon the primary infection but may evolve later. We have now simulated the evolution of R5 to X4 strains using a cloned NSI virus of the slowly replicating phenotype, that, upon prolonged culture in SUP-T1 cells, converted to the SI, highly replicating phenotype. The NSI, slowly replicating virus strain 168.1 was continuously passaged in SUP-T1 cells with or without AMD3100, a selective antagonist of CXCR4. After 120 days (24 passages) in culture, clear cytopathic effect (CPE) and formation of syncytia were noted in the untreated cell culture. Conversely, neither CPE nor syncytium formation were detected in the infected cells that had been cultured in the presence of AMD3100, despite low, but continuous virus replication. Virus recovered from the untreated cell cultures had expanded its coreceptor use from CCR5 to CXCR4 (R5X4 strain), whereas virus maintained in the presence of AMD3100 was solely of the R5 phenotype. DNA sequence analysis of proviral DNA isolated from untreated cells revealed the presence in the V3 loop of mutations that are indicative for the SI phenotype. No amino acid changes were noted in the V3 loop region of the proviral DNA isolated from AMD3100-treated cells even after 260 days in culture. Our results suggest that blockade of CXCR4 may prevent the emergence of fast replicating, T-cell tropic, SI strains of HIV. This, in turn, may have profound implications for the outcome of HIV infections.

5

Inhibition kinetics of HIV-1 integrase by polyhydroxylated aromatic derivatives, oligonucleotides and nucleotide analogues

E. Tramontano°, C. Musiu°, M. Artico*, R. Di Santo*, R. Costi*, S. Massa#, P. Franchetti†, M. Grifantini† and P. La Colla°.
Depts. of °Biologia Sperimentale, Univ. Cagliari, *Studi Farmaceutici, Univ. Roma, #Farmaco Chimico, Univ. Siena, †Scienze Chimiche, Univ. Camerino, Italy

Integration of human immunodeficiency type 1 (HIV-1) provirus is an essential step for viral replication. The virus-encoded enzyme that accomplishes this reaction, integrase (IN), is therefore a promising target for drug development. Recently, different classes of IN inhibitors have been identified. Among them are polyhydroxylated aromatics derivatives, phosphorotioate and G4-containing oligonucleotides, and nucleotide analogues. While active in enzyme assay, the majority of these compounds are unable to potently-selectively inhibit the HIV-1 replication in cell based assay. Therefore, for future drug development is particularly important to understand the mechanism of interaction between these derivatives and the HIV-1 IN. Here we report on the inhibition kinetics of the IN 3'-processing activity by different analogues of these three class of compounds using the Dixon kinetic analysis. Cinnamoyl-based derivatives were used as example of polyhydroxylated aromatics inhibitors and were found to compete with the DNA substrate used in the 3'-processing activity. Similarly, phosphorotioate oligonucleotides were competitive inhibitors of the DNA substrate. On the contrary, nucleotide analogues were found to inhibit the 3'-processing activity of the HIV-1 IN non-competitively with the DNA substrate, suggesting that the nucleotide binding site present on the HIV-1 IN may affect the catalytic site of the enzyme. Analogous studies on the strand-transfer activity of the HIV-1 IN are in progress.

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6

Processing of 5' Termini in Gapped Integration Intermediates by Human Immunodeficiency Virus Type 1 Integrase Accompanies Gap Repair

E. A. Faust, R. Wald, A. Shtevi, A. Acel, H. Triller, B. Udashkin and M. A. Wainberg Lady Davis Institute for Medical Research SMBD-Jewish General Hospital and McGill AIDS Center and Department of Medicine, McGill University, Montreal, Quebec, Canada

Classically, HIV-1 integrase is described as a dimer consisting of two identical subunits of 288 amino acids. With synthetic oligonucleotides as substrates, integrase catalyzes endonucleolytic cleavage, the so-called 3'-processing reaction, and polynucleotide transfer wherein integration or 3'-end joining, of the oligonucleotides occurs. These two sequential transesterification reactions require no exogenous energy source, but an appropriate metal cofactor, either Mn^{++} or Mg^{++} and the highly conserved tripartite amino acid D,D, 35E motif located in the integrase core domain. Completion of integration requires that twin 5-nucleotide gaps generated by 3'-end joining be repaired and that the unpaired 5' tails of the viral DNA be removed and joined to host cell DNA. Since the latter reactions have not been observed in vitro with dimeric forms of integrase it has been generally assumed that they are performed by host cell enzymes. We have reported previously the isolation of an alternative, low abundance multimeric form of HIV-1 integrase whose Mr=172,000 is consistent with a holoenzyme structure comprised of 5-6 identical integrase subunits. This form of HIV-1 integrase has DNA polymerase activity and has been shown to repair short gaps efficiently and completely. We now report that the integrase holoenzyme catalyzes a 5'-end processing reaction on gapped DNA substrates undergoing DNA repair. Virtually all of the repaired molecules are processed over time but no 5'-end joining was detected. The processing reaction which could result from the action of either a 5'-3' exonuclease or from an endonuclease activity of integrase, is dependent on such features of the DNA substrate as the unpaired 5'AC tail, and the integrity of the adjacent 5'TG/CA dinucleotide. Most importantly processing exhibits a virtual absolute requirement for amino acid E85 of integrase with a lesser dependence on amino acid E152. The amino terminal zinc finger domain is required as well. There is no apparent requirement for amino acids D116, D64 or E69. These novel reactions of integrase, namely DNA repair and 5'-end processing are attractive targets for the development of antiviral agents.

7

INHIBITION OF HIV-1 INTEGRATION AND INFECTIOUS VIRUS FORMATION BY CYTOPLASMIC Fab INTRABODIES AGAINST THE HIV-1 MATRIX PROTEIN,p17.

Reuven Levin [1], Abner M. Mhashilkar[2] and Wayne A. Marasco[3]

[1] Dept. of Infectious Diseases, Israel Institute for Biological Research, Ness Ziona, 74100, Israel. [2]Introgen Therapeutics Inc. Houston, TX. 77054 [3]Dept. of Cancer Immunology and AIDS. Dana-Farber Cancer Institute, Boston, MA. 02115, USA.

The HIV-1 matrix protein, p17, contains two subcellular localization signals that facilitate both nuclear import of the viral preintegration complex in non-dividing cells and infectious particle assembly at the plasma membrane of infected cells. The combined role of the p17 in both the afferent and efferent arms of the HIV-1 life cycle makes it a unique target for intracellular immunization base gene therapy strategies. We demonstrated, using a bicistronic expressor vector, that the genes encoding the rearranged heavy and light chains of an intracellular antibody, "Fab Intrabody", directed against a highly conserved carboxy terminal epitope of p17 of the Clade B HIV-1 genotype can inhibit HIV-1 infection when expressed in the cytoplasm of actively dividing $CD4^+$ T cells. Inhibition of infection occurs with both laboratory strains and syncytia- inducing primary isolates of HIV-1. When integration and infectious particle formation were examined separately, both processes were inhibited. This novel strategy of simultaneously blocking early and late events of the HIV-1 life cycle may prove useful in the clinical gene therapy setting for the treatment of HIV-1 infection and AIDS.

8

Characterization of the Activation Pathway of Phosphoramidate Triester Prodrugs of d4T and AZT

D. SABOULARD[1]*, L. NAESENS[1], C. BALLATORE[2], E. DE CLERCQ[1], C. MCGUIGAN[2] and J. BALZARINI[1]

[1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium and [2]Welsh School of Pharmacy, University of Wales, Cardiff, CF1 3XF, United Kingdom

The phosphoramidate triester prodrugs of 2',3'-dideoxynucleoside analogues (ddN) represent a convenient approach to bypass the first phosphorylation step to ddN-5'-monophosphate (ddN-MP), resulting in an improved formation of ddN-5'-triphosphate and, hence, higher anti-HIV efficacy [Balzarini *et al.*, Proc. Natl. Acad. Sci. USA, 93: 7295 (1996); McGuigan *et al.*, J. Med. Chem., 39: 1748 (1996)]. Surprisingly, while phosphoramidate derivatization of d4T markedly increases the anti-HIV activity in both wild-type and thymidine kinase-deficient CEM cells, the concept is far less successful for the AZT triesters. We now compared the metabolism of phosphoramidate prodrugs of d4T and AZT in crude CEM cell extract, mouse serum, human serum, and rat liver enzyme preparations. The first part in the activation pathway, consisting of the formation of the amino acyl ddN-MP metabolite, is mediated by carboxylesterases, and is inhibited by phenylmethylsulfonyl fluoride. The efficiency of this step was shown to be dependent on the amino acyl, alkyl and ddN moiety. Several triesters that showed no conversion to the amino acyl ddN-MP accumulated as the phenyl-containing intermediate and had poor, if any, anti-HIV activity. In contrast to the relative stability of the triesters in human serum, the conversion to the amino acyl ddN-MP metabolite was found to be remarkably high in mouse serum, due to high carboxylesterase activity. The subsequent conversion of the amino acyl ddN-MP metabolite to ddN-MP or ddN was most pronounced in rat liver microsomal preparations. While the triesters of d4T mainly resulted in d4T-MP release, the main metabolite formed from the AZT prodrugs was the free nucleoside. The rat liver enzyme involved in the formation of d4T-MP was shown to be distinct from alkaline phosphatase and phosphodiesterase, and proved to be inhibited by phosphocreatine and iodobenzene. We are currently isolating this phosphoamidase enzyme, and characterizing its substrate specificity.

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Response to Therapy and Safety Considerations in Treatment-Experienced HIV-Infected Patients on Abacavir (ABC) Salvage Therapy.

D MILDVAN, S NOWLING, F AHMED, S SHAH, R YARRISH. BETH ISRAEL MEDICAL CENTER, New York, NY, USA.

We evaluated the safety and virologic response of ABC (a new nucleoside RT inhibitor) in combination salvage therapy in 32 pts with advanced HIV infection (mean age 42.8 yrs). Pts were treated with a mean of 3.2 new agents, including ABC, in this open-label trial. Virologic response: Viral load data at 8 wks are available for 24 pts; of these, 7 (29%) were responders, with a $\geq$ 1-log drop in plasma HIV-1 RNA levels. Compared to non-responders, responders had lower baseline viral loads: mean 214,879 copies/mL vs. 419,899, respectively, and higher baseline CD4+ cell counts: mean 104.4 vs. 68.9 cells/μL, respectively. Mean CD4+ cell counts at week 8 were 128.6 and 71.2 cells/μL among responders and non-responders, respectively. Safety: Data are available for all 32 pts. Twelve pts. experienced adverse events, including rash (12), nausea/vomiting (6), fever (4), fatigue (1), insomnia (1). ABC hypersensitivity rxns (ABC-H: fever, flushing, rash) were considered likely in one pt and possible in 2; the latter 2 pts had ABC discontinued without ill effects. The pt. with probable ABC-H had concomitant adrenal insufficiency and developed fever and respiratory distress after a short interruption, then resumption of ABC. The pt. died of other AIDS-related events 2 months later. The remaining 9 pts with rash had transient non-progressive symptoms and were taking other agents that may have produced them: nonnucleoside (nn)RTIs 8, IFN-alpha 1, ritonavir 1. 8/9 rashes were "treated through" (2/8 with steroid taper); 1/9 had both ABC and delavirdine (DLV) interrupted: when re-exposure to DLV was found to exacerbate the rash, it was discontinued and ABC was uneventfully resumed. When pts with and without rash were compared with respect to concomitant nnRTI usage, it was found that 11/12 pts with rash (92%) and 13/20 without rash (65%) were receiving nnRTIs with ABC (ns). Conclusions: These data demonstrate a 29% response rate among treatment-experienced, advanced pts with combination ABC salvage therapy, representing an important new therapeutic option. ABC-H occurs infrequently and can be distinguished from more common adverse events by close patient monitoring.

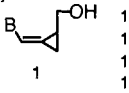
Oral Session II: Herpesvirus Infections I

10

ENANTIOSELECTIVITY OF THE ANTIVIRAL EFFECT OF METHYLENECYCLOPROPANE NUCLEOSIDE ANALOGUES.

J. Zemlicka[1], Y.-L. Qiu[1], T. Kira[2], E. Gullen[2], Y.-C. Cheng[2], R. G. Ptak[3], J. M. Breitenbach[3], J. C. Drach[3], C. B. Hartline[4], E. R. Kern[4].
[1]Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI; [2]Yale University School of Medicine, New Haven, CT; [3]School of Dentistry, University of Michigan, Ann Arbor, MI; [4]University of Alabama School of Medicine, Birmingham, AL.

Racemic methylenecyclopropane analogues 1a - 1f are broad-spectrum anti-herpesvirus agents that are also effective against MCMV in vivo. The R- and S-enantiomers of 1a - 1f were synthesized and their antiviral activity against HCMV, EBV, VZV, HSV-1 and HSV-2 in vitro was investigated. The enantioselectivity of the antiviral effect depends on the type of the heterocyclic base, virus and host cells involved. Thus, R- and S-enantiomers of adenine analogue 1a were equipotent or almost equipotent against HCMV (Towne, EC_{50} 2.9 and 2.4 μM; AD 169, EC_{50} 6.9 and 1.9 μM) and VZV (EC_{50} 1.5 μM for both enantiomers) but the S-enantiomer was strongly preferred against MCMV (EC_{50} 0.55 vs. 55 μM). The same preference was found for 1b - 1f against HCMV (EC_{50} 1.8 - 21 μM vs. 30 - >100 μM) and VZV. The S-enantiomers of 1b - 1f were also



1a: B = adenine
1b: B = guanine
1c: B = 2-amino-6-chloropurine
1d: B = 2,6-diaminopurine
1e: 2-amino-6-cyclopropylaminopurine
1f: 2-amino-6-methoxypurine

favored in EBV assays although enantioselectivity varied with the type of assay. By contrast, 1a exhibited a clear R-selectivity in H-1 cells (EC_{50} 0.09 vs. 0.63 μM) which was less pronounced in Daudi cells (EC_{50} 3.0 vs. 7.8 μM). The R- and S-enantiomers of 1c were equipotent in H-1 cells (EC_{50} 2.3 and 1.3 μM). The S-enantiomers were the most effective agents in HSV-1 and HSV-2 assays of active analogues. It is likely that these results reflect differences in mechanism of phosphorylation and/or affinities of the corresponding triphosphates for target viral polymerases. Supported by NIH grants RO1-CA32779, RO1-AI33655, RO1-38204, U19-AI31718, RO1-AI36872 and NO1-AI-35177.

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Anti-HCMV Activity of Selected Compounds in Human Retinal Pigment Epithelial (RPE) Cells

G. ANDREI,[1] R. SNOECK,[1] D. SCHOLS,[1] E. DE CLERCQ,[1] B. BODAGHI[2] and S. MICHELSON[2]
[1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium and [2]Institut Pasteur, F-75724 Paris, France

Human cytomegalovirus (HCMV) is a common opportunistic infection resulting in retinitis in 15-40% of AIDS patients. It is the leading cause of AIDS-associated blindness. The human retina is a multistratified tissue composed of ten different layers. The nine inner layers form the neural retina, while the outer layer is composed by the retinal pigment epithelium (RPE). Earlier reports showed that RPE cells are fully permissive for HCMV infection *in vitro*. However, viral replication is significantly slower in these cells in comparison with the human embryonic lung (HEL) fibroblasts that are widely used to propagate HCMV *in vitro*. The aim of the present study was to evaluate the antiviral activity of reference anti-HCMV compounds, i.e. cidofovir (CDV), ganciclovir (GCV), foscarnet (PFA) and the new anti-HCMV drug 2-chloro-3-(3-pyridyl)-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV 423) in RPE cell cultures. Confluent RPE cells were infected with HCMV (AD-169 strain) at different multiplicities of infection and various concentrations of the test compounds were added after virus adsorption. Compounds remained in contact with the cells for 10 days and then were replaced by medium without drugs. Cells were harvested at 21 days after infection and analyzed by flow cytometry for expression of immediately early (IE) and early (E) antigens by using monoclonal antibodies E13 and ccH2, respectively. The 50% inhibitory concentration (IC_{50}), or concentration required to inhibit antigen expression by 50%, was calculated for each drug. IC_{50} values obtained in RPE cells ranged from 0.05 to 0.112 µg/ml for CDV, 0.05 to 0.13 for GCV, 5 to 11 µg/ml for PFA and 0.05 to 0.3 µg/ml for CMV 423, these IC_{50} values being comparable to those obtained in HEL cells.Thus, RPE cells can be used as an *in vitro* system to evaluate the antiviral activity of anti-HCMV drugs.

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In Vitro Inhibition of HCMV Replication by the tetrahydro-indolizine RPR111423

R. SNOECK,[1] G. ANDREI,[1] C. NEMECEK,[2] E. DE CLERCQ[1] and C. ROY[2]

[1]Rega Institute for Medical Research, K.U.Leuven, B-3000 Leuven, Belgium; [2]Rhône-Poulenc Rorer, Vitry/Seine, France

RPR111423 and its derivatives belong to a new class of compounds, the tetrahydroindolizine derivatives, with potent activity against human cytomegalovirus (HCMV) that act by an entirely novel mechanism of action. RPR111423 was evaluated for its anti-HCMV activity against laboratory (AD-169 and Davis strains) and clinical HCMV strains isolated from AIDS patients, transplant recipients, and children with congenital HCMV infection. When the different clinical isolates were tested for their susceptibility to RPR111423 by inhibition of viral plaque formation in human embryonic lung (HEL) cells, the IC_{50} (50% inhibitory concentration) was 0.0047 ± 0.0036 μM compared to 0.157 ± 0.125 μM and 1.018 ± 0.777 μM for cidofovir (CDV) and ganciclovir (GCV), respectively. The potent anti-HCMV activity was also confirmed by a virus yield reduction assay employing both reference and clinical strains at different multiplicities of infection. The CC_{50} (50% cytotoxic concentration) for RPR111423 was 72.4 μM based on either alteration of cell morphology or inhibition of cell growth, the selectivity index (CC_{50}/IC_{50} ratio) being 15,404. RPR111423 retained activity against GCV- and CDV-resistant (GCV^r, CDV^r) AD-169 mutant strains selected *in vitro*, and against clinical isolates of GCV^r or GCV^r/CDV^r HCMV obtained from patients receiving GCV therapy. Due to its potent and selective activity against HCMV isolates, whether resistant or not to current anti-HCMV drugs, RPR111423 represents an attractive potential candidate for the treatment of HCMV infections.

13

Specific inhibition of immediate early antigen expression by antisense oligonucleotides (ISIS 2922) prevents the up-regulated IL-8, GRO-alpha and ICAM-1 production in HCMV-infected fibroblasts.

R Kotchetkov, E. Weimer, J.-U. Vogel, H.-W. Doerr, J Cinatl Jr.

Department of Medical Virology and Department of Children Hematology and Oncology, Johann Wolfgang Goethe-University-Clinic, Frankfurt am Main, Germany

In vitro and *in vivo* studies demonstrated that HCMV-stimulated cytokine production and/or up-regulation of adhesion molecules on surfaces of infected cells are associated with inflammatory responses. In the present study we observed the different influence of ganciclovir (GCV), foscarnet (PFA) and a phosphorothioate oligonucleotide (ISIS 2922) complementary to HCMV immediate-early (IE) RNA on HCMV induced secretion of C-X-C chemokines (IL-8 and GRO-alpha) and intercellular adhesion molecule 1 (ICAM-1). As compared with mock-infected cells In HCMV-infected fibroblast cells at a multiplicity of infection 1 were the IL-8 and GRO-alpha up to 8-fold increased and ICAM-1 was 4-fold up-regulated. Treatment of the cells with GCV (50 μM) or PFA (400 μM) completely suppressed virus replication as demonstrated by late (L) antigen or infectious virus production. These both drugs did not influence IE antigen expression and had no effects on HCMV-induced cellular changes in IL-8, GRO-alpha and ICAM-1 levels. The ISIS 2922 at a concentration of 1 μM suppressed both IE and L antigen by 99% and inhibited infectious virus production by 1000- 10000 fold. Moreover, ISIS 2922 completely suppressed HCMV-induced up-regulation of both C-X-C chemokines and ICAM-1. The results showed that HCMV induced pathways involved in inflammation can not be inhibited by drugs targeted on virus DNA synthesis. In contrast agents such as antisense oligonucleotides specifically inhibiting IE antigen expression prevent up-regulation of both C-X-C chemokines and ICAM1 suggesting that HCMV IE expression accounts for these pathogenic effects

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Pyrrolidine-5,5-Translactams, Novel Herpes Protease Inhibitors that are potent Antivirals against Human Cytomegalovirus

A.D. Borthwick*, A. Crame, A.M. Exall, D.L. Jackson, A.M. Mason, A.M.K.Pennell, G.G, Weingarten, T.M. Haley, G.J. Hart and N.R.. Parry

Glaxo Wellcome Research and Development, Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts SG1 2NY. UK.

Human herpes viruses encode a serine protease which is essential for viral replication. Recent X-ray structures of the serine proteases of HCMV, HSV-1, HSV-2 and VZV revealed that these enzymes belong to a novel class of serine proteases where the active site is composed of the His, His, Ser triad. Substrate cleavage sites across all the herpes virus family are unique and highly conserved and these enzymes have become attractive molecular targets for the design of novel antiviral drugs. We recently reported a series of monocyclic β-lactams as mechanism based inhibitors of human cytomegalovirus protease (Borthwick *et al., Bioorg. Med. Chem. Lett.*, 1998). We now report on the design and synthesis of a novel class of bicyclic pyrrolidine-5,5-translactams as potent herpes protease inhibitors. These are selective over the mammalian proteases with good plasma stability and are potent as antivirals against human cytomegalovirus. The SAR, mechanism of inhibition of HCMV protease and antiviral activity of this new class of serine protease inhibitors will be discussed.

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Non-Nucleoside Pyrrolopyrimidines With a Unique Mechanism of Action Against Human Cytomegalovirus. J.G. Jacobson, T.E. Renau, M.R. Nassiri, D.G. Sweier, J.M. Breitenbach, L. B. Townsend and J.C. Drach. University of Michigan, Ann Arbor, Michigan 48109 U.S.A.

From a prior study in which we evaluated series of non-nucleoside pyrrolo[2,3-*d*]pyrimidines as inhibitors of human cytomegalovirus (HCMV) (Renau *et al. J. Med. Chem.*, 39:873-880 & 3470-3476 1996) we have selected three active analogs for detailed study. In an HCMV plaque reduction assay, these compounds designated UMJD 828, 951 and 1028 had IC_{50}'s of 0.4–1.0 μM. Similar results were obtained when UMJD 828 and 951 were examined by HCMV ELISA (IC_{50}'s =1.9 and 0.4 μM, respectively) and when UMJD 828 was tested in a viral DNA-DNA hybridization assay (IC_{50} = 1.3 μM). In yield reduction assays at a low multiplicity of infection (MOI), all three compounds produced multiple $\log_{10}$ reductions in virus titer that were comparable to the activity of ganciclovir (GCV, IC_{90}= 0.2 μM). In contrast to GCV, the reduction of viral titers by UMJD 828, 951, and 1028 decreased with increasing MOI. Cytotoxicity in human foreskin fibroblasts and KB cells ranged from 32 to >100μM. In addition, UMJD 828 (the only compound tested) was less toxic against human bone marrow progenitor cells than GCV. Time of addition and time of removal studies established that the three pyrrolopyrimidines inhibited HCMV prior to the effect of GCV on viral DNA synthesis but after viral adsorption. UMJD 828 was equally effective against GCV-sensitive and GCV-resistant HCMV clinical isolates. Combination studies with UMJD 828 and GCV showed that the effect of the two compounds on HCMV was additive but not synergistic. Taken together, the data indicate that these pyrrolopyrimidines target a viral protein required in a MOI-dependent manner and expressed early in the HCMV replication cycle. Supported by N.I.H. Research Contract N01-AI72641 and grant U19-AI31718 for a National Cooperative Drug Discovery Group for Opportunistic Infections; and by research funds from the University of Michigan.

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Induction of the Epstein-Barr Virus Thymidine Kinase- Sensitivity of Viral Thymidine Kinase-Expressing Cells to Nucleoside Analogs. S.M. Moore, F.M. Hamzeh, and R.F. Ambinder. Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

The presence of Epstein-Barr virus (EBV) in the tumor cells of some EBV-associated malignancies may facilitate selective killing of these tumor cells. Expression of the lytic gene, thymidine kinase (TK), may render cells sensitive to nucleoside analogs such as ganciclovir. We have investigated the induction by 5-azacytidine of the EBV TK in EBV(+) Rael cells, a Burkitt's lymphoma derived cell line. After treatment with 0.5 μM 5-azacytidine for six hours, immunohistochemical analysis showed immediate early lytic antigen expression in 60 – 75% of the treated cells after twenty-four hours. A dose-dependent induction of the viral TK was demonstrated by immunoblotting using a polyclonal rabbit antiserum raised against a peptide of the EBV TK. In order to determine whether expression of the viral TK might render EBV-infected cells susceptible to killing by antiviral nucleoside analogs, we created stable cell lines expressing the EBV TK. These cell lines, but not control cell lines, were selectively sensitive to ganciclovir (GCV), penciclovir (PCV), bromovinyldeoxyuridine (BVdU), and 3'-azido-3'-deoxythymidine (AZT) whereas neither EBV TK-expressing cells nor control cells were sensitive to acyclovir (ACV). When 5-azacytidine TK induction was combined with nucleoside analog treatment, we observed a synergistic killing of Rael tumor cells but not EBV(-) Burkitt's lymphoma cells. Furthermore, stable EBV TK-expressing cells and 5-azacytidine induced Rael cells were shown to phosphorylate GCV to a much greater extent than control cells. Together, these data show that ganciclovir is a substrate of the EBV TK and indicate that GCV, as well as PCV, AZT, and BVdU, are activated by the EBV TK such that cells expressing the viral TK are specifically targeted. Pharmacologic induction of the EBV TK in combination with a nucleoside analog may be a useful therapeutic strategy for the treatment of EBV-associated malignancies.

Oral Session III: Mini-Symposium – No Abstracts

Oral Session IV:
Respiratory Virus Infections, Emerging Infections

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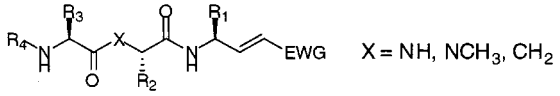
Structure-Based Design and Assay of Irreversible Human Rhinovirus 3C Protease Inhibitors. S. A. Fuhrman, P. S. Dragovich, S. E. Webber, A. K. Patick, D. A. Matthews, C. A. Lee, T. Tuntland, L.S. Zalman, T. J. Prins, J. T. Marakovits, R. Zhou, J. Tikhe, C. E. Ford, J. W. Meador, J. E. V. Harr, M. B. Kosa, R. A. Ferre, E. L. Brown, S. L. Binford, M.A. Brothers, D. M. DeLisle, and S. T. Worland. Agouron Pharmaceuticals, Inc., 3565 General Atomics Court, San Diego, CA 92121 USA.

The development of peptidyl and peptidomimetic human rhinovirus (HRV) 3C protease (3CP) inhibitors which incorporate various Michael acceptor moieties is described. These compounds irreversibly bind to the active site 3CP cysteine residue and exhibit potent antiviral activity against multiple rhinovirus serotypes in cell culture. The identification of a ketomethylene-containing 3CP inhibitor (AG7088) as a pre-clinical development candidate is detailed along with a comparison between several tripeptidyl and *N*-methylated tripeptidyl compounds.



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VX-497, a novel IMPDH inhibitor, is a broad spectrum antiviral agent with superior activity compared to ribavirin against selected DNA and RNA viruses *in vitro*. A.D. Kwong. Vertex Pharmaceuticals Inc., 130 Waverly St., Cambridge MA 02139 USA.

The enzyme inosine 5'-monophosphate dehydrogenase (IMPDH) catalyzes an essential step in the *de novo* biosynthesis of guanine nucleotides, namely the conversion of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP). The major event occurring in cells exposed to competitive IMPDH inhibitors such as ribavirin or uncompetitive inhibitors such as MPA, is a depletion of the intracellular GTP and dGTP pools. Ribavirin is approved as an inhaled antiviral agent for treatment of respiratory syncytial virus (RSV) and orally, in combination with interferon α, for the treatment of chronic hepatitis C virus (HCV) infection. VX-497 is a potent, reversible uncompetitive IMPDH inhibitor which is structurally unrelated to other known IMPDH inhibitors. Studies were performed to compare the cytotoxic effect and antiviral efficacy of VX-497 and ribavirin against a wide variety of DNA viruses (hepatitis B virus (HBV), human cytomegalovirus (HCMV) and herpes simplex virus type 1(HSV)) and RNA viruses (respiratory syncytial virus (RSV), parainfluenza-3 virus, bovine viral diarrhea virus (BVDV), Venezuelan equine encephalomyelitis virus (VEE), dengue virus, yellow fever virus (YFV), coxsackie B3 virus and influenza A virus). VX-497 was 18- to 186-fold more potent than ribavirin against HBV, HCMV, RSV, HSV, parainfluenza-3 virus, and VEE viral infections in cultured cells. The therapeutic index for VX-497 was significantly better than that of ribavirin for HBV and HCMV (14- and 39-fold, respectively). These data are supportive of the hypothesis that VX-497, like ribavirin, is a broad-spectrum antiviral agent and furthur evaluation of VX-497 in antiviral applications appears to be warranted.

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Influence of Treatment Schedule and Viral Challenge Dose on the *In Vivo* Influenza-Inhibitory Effects of the Orally Administered Neuraminidase Inhibitor GS4104.

R. W. Sidwell, K. W. Bailey, P. A. Bemis, M. H. Wong, and J. H. Huffman. Inst. for Antiviral Research, Utah State Univ., Logan, UT USA.

Previous studies have shown that twice daily oral gavage (p.o.) treatment with (3*R*, 4*R*, 5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid (GS4104) was strikingly inhibitory to infections in mice induced by influenza viruses. These data correlated well with the murine serum half-life of GS4104 as reported by Li et al. (Antimicrob Ag. Chemother. 42:647-53, 1998). It was of interest to determine how alteration of p.o. treatment schedule of a dose of 5 mg/kg/day of this compound to 1, 3, or 4 times daily treatments or to therapy once only at varying times relative to virus exposure would affect the infection in mice induced by influenza A (H1N1). All treatments of 2 or greater per day were highly inhibitory to this infection, unless the therapy was terminated relatively early in the infection (days 2-3) in which case efficacy was curtailed. These data indicated there was a requirement for the drug to be in the host when lung virus titers were reaching maximal levels and that at least a twice daily therapy is needed to maintain adequate serum levels to achieve an appropriate antiviral effect. Single administration of the compound was essentially not effective at any time during the infection. Twice daily p.o. treatment for 5 days with 20 mg/kg/day of GS4104 totally prevented deaths in mice receiving high viral challenge doses which were sufficient to kill placebo-treated animals in less than 5 days. Other parameters of antiviral efficacy (lung consolidation, arterial oxygen saturation, lung virus titers, serum immunosuppressive acidic protein) were also markedly inhibited regardless of viral challenge dose. These data provide further insights into how a maximum therapeutic benefit can be derived from the use of this orally effective influenza virus neuraminidase inhibitor. (Supported by contract NO1-AI-65291 from the Virology Branch, NIAID, NIH).

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Oral administration of GS 4104 protects chickens against death due to infection with a highly pathogenic avian influenza virus.

D.B. Mendel[1], L.V. Gubareva[2], H.R. Wiltshire[3], and R.G. Webster[3]. [1]Gilead Sciences, Foster City, CA, USA. [2]St. Jude Children's Research Hospital, Memphis, TN, USA. [3]Roche Discovery Welwyn, Welwyn Garden City, Hertfordshire, UK.

The majority of avian influenza viruses are nonpathogenic, replicate in the gastrointestinal (GI) and respiratory tracts of infected birds, and, like the mammalian viruses, are not lethal. In contrast, the highly pathogenic avian influenza (HPAI) viruses cause systemic infections which are generally lethal. The goal of this study was to determine whether oral administration to chickens of GS 4104, a prodrug of the influenza neuraminidase inhibitor GS 4071 which is currently under clinical development for the prevention and treatment of influenza virus infections in humans, could produce sufficient systemic exposure to GS 4071 to protect against death due to infection with a HPAI virus. Oral GS 4104 treatment caused a dose-dependent increase in survival time and survival rate of chickens infected with the influenza A/Chick/Victoria/1/85 (H7N7) virus, with a 60% survival rate in animals given 100 mg/kg of GS 4104 twice daily for 5 days. Consistent with the observation that GS 4104 did not completely prevent virus replication in the respiratory or GI epithelium, all the GS 4104-treated animals that survived the initial infection survived a rechallenge with the same virus in the absence of treatment, indicating that the GS 4104-treated animals developed an immune response to the virus. Pharmacokinetic studies indicated that a high dose of GS 4104 was needed for efficacy because the bioavailability of GS 4071 from orally administered GS 4104 is low in chickens (~5%) compared to man (~80%) and other species. The systemic exposure to GS 4071 in chickens given the 100 mg/kg bid dose of GS 4104 was similar to that seen in humans given a dose (75 mg bid orally) which has been shown to be efficacious for the treatment of influenza virus infections in humans and which is undergoing further clinical evaluation.

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A Unique Animal Model for the Study of the Pathogenesis and Therapy of Flavivirus Infection

P. LEYSSEN, A.-M. VANDAMME, S. VAN DOOREN, E. DE CLERCQ and J. NEYTS

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Flaviviruses such as Dengue Fever Virus (DFV), Tick Borne Encephalitis Virus (TBEV), Japanese Encephalitis Virus (JEV), Yellow Fever Virus (YFV), West-Nile Fever Virus are responsible for life-threatening infections in man. Yet, no specific antiviral therapy is available for the treatment of these virus infections. There is also no small animal model for the study of the pathogenesis of flavivirus infections or for the *in vivo* evaluation of potential new therapies. We have now used the murine flavivirus Modoc to establish in small laboratory animals, a model for flavivirus infections. Modoc virus replicates well in Vero cells and causes a typical cytopathic effect. Replication of the virus was confirmed by means of RT-PCR using primers specific for DFV. In Vero cell cultures Modoc virus appeared equally sensitive to various antiviral agents (ribavirin, EICAR, MPA and tiazofurin) as the human viruses YFV and DFV. Intraperitoneal and intranasal infection of SCID mice, but not immunocompetent NMRI mice, resulted in 100% mortality within a time-span of two weeks. All animals died of encephalitis. With the aid of RT-PCR, replicating virus was detected in brain, spleen, lung and salivary glands of infected animals. Next, hamsters were inoculated with Modoc virus: from day 4 after infection the virus was shed in the urine of the infected animals. This allowed to monitor in the same animal, over a time-span of about two weeks, and by means of a non-invasive method, the potential *in vivo* activity of antiviral agents. The genome of Modoc virus is currently being sequenced. At present, almost the entire sequence of the RNA-dependent RNA polymerase has been determined. High similarity at the nucleic acid level with DFV type 1 and 2 (dependent on the region up to 82% homology), YFV, JEV and TBEV has been found. Thus, Modoc virus may be a particular attractive model virus to evaluate the pathogenesis and therapy of flavivirus infections. It may also be used in the construction of chimeric viruses (e.g. Modoc/DFV or Modoc/HCV) that replicate and cause morbidity and mortality in small laboratory animals.

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Treatment of Lethal Filovirus or Orthopoxvirus Infection in Mice with One or More Large Doses of an S-adenosylhomocysteine Hydrolase Inhibitor. M Bray, E Thompson, JW Huggins. Virology Division, USAMRIID, Fort Detrick, Frederick, MD, USA

There is currently no effective treatment for infection with Ebola Zaire virus, which causes severe or fatal hemorrhagic fever in humans and nonhuman primates. However, the virus is sensitive to inhibition *in vitro* by a class of broad-spectrum antiviral agents, the S-adenosylhomocysteine (SAH) hydrolase inhibitors, which are believed to block methylation of the 5' cap of viral messenger RNA through a feedback mechanism. We recently adapted Ebola Zaire virus to uniformly lethal virulence in adult, immunocompetent mice. Using this model, we found that up to 100% of mice survive Ebola infection when treated for 9 days with thrice-daily doses of 2.2 to 20 mg/kg of the SAH hydrolase inhibitor carbocyclic 3-deaza-adenosine, starting on the day of or the day after a 30 LD_{50} challenge. Up to 90% survived if treatment was began on day 2, and 40% if begun on day 3, when mice began to show signs of Ebola infection. We now report that a single large injection of the same or a related compound is as effective as multidose therapy. One injection of 80 mg/kg of carbocyclic 3-deazaadenosine on day 1 or day 2 postinfection protected 100% of mice challenged with 300 LD_{50} of Ebola virus, and up to 66% treated on day 3. A dose of 1 mg/kg of a more potent compound, 3-deazaneplanocin A, protected all mice treated on day 1 or day 2 and up to 100% of mice treated on day 3. Treatment markedly reduced viremia. This dose of drug caused no apparent hepatic or renal toxicity. We have also evaluated this treatment regimen in a uniformly lethal model of cowpox virus infection of the respiratory tract in immunocompetent mice. One dose of 80 mg/kg of carbocyclic 3-deazaadenosine on the day of a 100 LD_{50} intranasal challenge protected 50% of mice against death. Survival was increased to 90-100% by giving additional doses on day 3 or on days 3 and 5. Combination therapy with HPMPC (cidofovir) and one dose of either carbocyclic 3-deazaadenosine or 3-deazaneplanocin A improved survival over HPMPC alone.

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Low-Dose Ritonavir (RTV) Compared with Standard-Dose RTV in Protease Inhibitor (PI)-Naive Subjects

M. Giordano, Cornell Univ. Med. College, New York, NY, US; G. Beall, Harbor-UCLA Medical Center, Torrance, CA, US; M. Sension, No. Broward Co. Hosp., Ft. Lauderdale FL, US; D. Parenti, George Washington Univ., Washington, D.C., US; P. Keiser, UT Southwestern, Dallas, TX, US; E. Yakim, IMS, Bethesda, MD, US;

Convenient, well-tolerated BID antiviral (AV) regimens aid patient compliance. We report results of a multicenter, open-label, comparative trial of standard high-dose (HD) RTV 600mg BID with low-dose (LD) RTV 400mg BID in minimally AV-experenced, PI-native, HIV-infected pts. The primary endpoint was the proportion of pts with undetectable viral load (VL) at wk 12. The study was powered to detect a 50% difference between LD and HD RTV. N=67 patients, aged 18-55 yrs, were included. Baseline domographics: Median age 37.1 yrs; 24% black, 30% Latino, 31% white; 79% M, 21% F. Median CD4 cells: 325HD, 218LD; median VL (c/mL): 55,040 HD; 87,530 LD. Pts were randomized to LD (n=36) or HD (n=31), with d4T and 3TC. Pts with VL>400 at wk 12 added saquinavir (SQV). Results: 23/25 (92%) HD and 26/33 (79%) LD pts were undetectable after 12 wks (p>n.s.). After 24 wks 15/17 (88%) HD and 18/20 (90%) LD pts remained undetectable. More LD (3/30 (10%)) than HD (1/28 (3.6%)) added SQV at wks 16-24. Compliance was equal in both groups (HD 95% and LD 96%) though more HD pts reduced dosage (6 vs 1). AE >Grade II were more frequent in HD (17) than LD (4) pts. Conclusions: Reduced-dose RTV is better tolerated than standard-dose RTV and does not result in a significant reduction in the proportion of pts whose VL is undetectable after 12 wks. Comparable results are exected at wk 24 and will be reported. Therefore, LD RTV is a reasonable treatment for PI-naive pts if VL is followed and intensification with SQV is allowed.

Oral Session V: Retrovirus Infections II

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Design, Synthesis, and Enzymology of Potential Inhibitors of HIV Integrase

V. Nair,* M. Taktakishvili, and B. Seo, Department of Chemistry, The University of Iowa, Iowa City, Iowa 52242, USA

The viral enzyme, HIV integrase, is involved in the integration of viral DNA into host cell DNA. Recognition by this enzyme of viral DNA involves specific sequences (5′-ACTG...CAGT-3′). Endonuclease activity specifically removes two nucleotides from each end of double helical viral DNA (3′-processing) to produce new 3′-hydroxyl ends (CAOH-3′) in the first step of integrase action. This truncated viral DNA is coupled in the next steps to host cell DNA (integration) which includes the DNA strand transfer reaction. We have investigated the synthesis of mono- and higher nucleotides as inhibitors of HIV integrase and this is the emphasis of the presentation. The design of potential inhibitors includes molecules with modifications in both the base and carbohydrate moieties. Results of the synthetic work will be described. Structural data presented and explained will include 2D NMR, hypochromicity, and circular dichroism. Integrase inhibition studies will be mentioned. Comparisons will be made between the inhibition activities of these compounds and those of other nucleotides and oligonucleotides.

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Dendrimers Inhibit the Replication of Human Immunodeficiency Virus (HIV) by a Dual Mechanism of Action.

M. Witvrouw,[1] C. Pannecouque,[1] B. Matthews,[2] D. Schols,[1] G. Andrei,[1] R. Snoeck,[1] J. Neyts,[1] P. Leyssen,[1] J. Desmyter,[1] J. Raff,[2] E. De Clercq[1] and G. Holan[2]
[1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium, [2]Biomolecular Research Institute, Bayview Ave., Clayton Vic 3168, Australia

Polyanionic dendrimers were synthesized and evaluated for their antiviral effects. Phenyl dicarboxylic acid (BRI6195) and naphthyl disulfonic acid (BRI2923) dendrimers were found to inhibit HIV type 1 (HIV-1 strain III_B) replication in MT-4 cells at a concentration of 0.03 and 0.3 μg/ml, respectively. The dendrimers were not toxic to MT-4 cells up to the highest concentration tested (250 μg/ml). The dendrimers were also effective against various other HIV-1 strains, including clinical isolates and HIV-1 strains resistant to reverse transcriptase inhibitors, HIV-2 strains and simian immunodeficiency virus (SIV strain MAC_{251}). HIV strains that contained mutations in the *env* glycoprotein gp120 (engendering resistance to known adsorption inhibitors) also displayed reduced sensitivity to the dendrimers. Moreover, BRI6195 and BRI2923 proved inhibitory to human cytomegalovirus strain Davis (EC_{50}: 0.2 and 1.0 μg/ml) and strain AD-169 (EC_{50}: 6 and 15 μg/ml), herpes simplex virus type 1 (HSV-1 strain KOS) (EC_{50}: 0.4 and 0.6 μg/ml) and type 2 (HSV-2 strain G) (EC_{50}: 0.4 and 1.9 μg/ml), thymidine kinase deficient (TK^-) HSV-1 (EC_{50}: 9.6 μg/ml), vesicular stomatitis virus (EC_{50}: 16 and 48 μg/ml), yellow fever virus (EC_{50}: 166 and 16.8 μg/ml), reovirus type 1 (EC_{50}: 80 μg/ml) and Dengue fever virus (EC_{50}: >200 and 89 μg/ml), respectively. An HIV-1 strain, showing cross-resistance to known virus adsorption inhibitors, was selected in MT-4 cells in the presence of increasing concentrations of BRI6195. Mechanism of action studies indicated that the naphthyl disulfonic acid dendrimer (BRI2923) can interact at two stages of the HIV-1 replicative cycle: virus adsorption and reverse transcription.

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Long Lasting and Efficient Inhibition of HIV Replication in Human Primary Macrophages by treatment with Protease Inhibitors.

CF. Perno, S. Aquaro, T. Guenci, P. Bagnarelli*, M. Clementi[§], A. Cenci, E. Balestra, R. Caliò

Dept. of Experimental Medicine University of Rome 'Tor Vergata'; *University of Ancona; [§]University of Trieste; Italy.

Control of HIV replication in macrophages (M/M) represents a crucial achievement for all therapeutic approaches. Once infected, M/M become insensitive to drugs acting at early stages of virus replication. For this reason, protease inhibitors (P.I.) represent the only drugs currently available potentially effective upon these chronically-infected cells. Objective of the study was than to assess whether two P.I., amprenavir and ritonavir, are able to perturb virus dynamics in macrophages. Human primary M/M were infected in vitro with a M-tropic HIV strain (HIV-1_{Ba-L}). Virus replication in the presence or absence of P.I. was assessed by quantitative PCR for unspliced and multiply spliced transcripts of HIV, by p24 gag protein production in the supernatants, and by virus titration. Results were compared to those obtained in peripheral blood lymphocytes (PBL) under the same experimental conditions. HIV replication in M/M shows a dynamics totally different than that of PBL, with a peak of production of HIV-RNA and proteins at day 14 (later but in the same range than that achieved in PBL) followed by a plateau lasting at least 50 days. Treatment with P.I. is totally unable to affect RNA transcripts, but strongly decreases the production of mature virus particles at concentrations above 1 μM, i.e. about 25 fold greater than those active in PBL or in macrophages treated with P.I. before virus challenge. Inhibition of virus production in chronically infected M/M by P.I. was maintained for at least 2 weeks of treatment, however drugs withdrawal causes an immediate restart of production of mature virus proteins. Nevertheless, virus titration shows that the production of infectious virus particles is maintained more one log lower than that of control M/M even one week after drug removal. In conclusion, HIV production in M/M (far greater than in PBL) can be controlled by P.I. only at concentration 25 fold greater than those active in PBL. This suggests that antiviral effect of P.I. may be different in body compartments, such as central nervous system, where M/M are the major target of HIV.

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Role of macrophage protection in the development of murine AIDS

M. Magnani, A. Fraternale, A. Tonelli, A. Casabianca, G. Vallanti, L. Chiarantini, [1]G. F. Schiavano, [2]A. Carnevali, [3]U. Benatti, [3]A. De Flora .

Istituto di Chimica Biologica "G. Fornaini", [1]Istituto di Igiene, Università di Urbino, 61029 Urbino; [2]Anatomia Patologica, Ospedale S. Salvatore, 61100 Pesaro; [3]Istituto di Chimica Biologica, Università di Genova, 16132 Genova, Italy

Macrophages have a key role in AIDS pathogenesis and thus the controlling of infectivity and viral replication in these cells is a key issue in any antiretroviral therapy. Using a murine model of AIDS, we evaluated new therapeutic approaches specifically designed for the protection of macrophages. We took advantage of the unique ability of autologous erythrocytes to selectively deliver drugs to macrophages. The antiviral drugs selected were a new homodimer of AZT ($AZTp_2AZT$) and reduced glutathione (GSH). The addition of an oral drug for the protection of lymphocytes (AZT) was also investigated. C57BL/6 mice infected with LP-BM5 were treated with GSH-loaded erythrocytes, GSH-loaded erythrocytes plus oral AZT, or GSH/$AZTp_2AZT$-loaded erythrocytes plus oral AZT. The treatments including AZT and erythrocytes loaded with GSH alone or with GSH plus $AZTp_2AZT$ provided similar results and were the most effective in inhibiting the progression of MAIDS: they significantly reduced all signs of the disease and BM5d proviral DNA content in infected organs. Treatment with GSH-loaded erythrocytes gave significant results for only the reduction in proviral DNA content. The results reported in this paper confirm the important role of macrophages in retroviral infection and moreover prove that erythrocytes, by selectively protecting these cells, strongly affect MAIDS progression. Furthermore, the combination of GSH- or GSH/$AZTp_2AZT$-loaded erythrocytes with an oral nucleoside analogue (AZT) for the protection of lymphocytes, provides additive responses in all the parameters investigated.

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Potent and selective inhibition of HIV-1 replication by a novel Tat antagonist, EM2487, in acutely and chronically infected cells

M. Baba, M. Okamoto and H. Takeuchi* *Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520 and *Department of Exploratory Drug Research, Eisai Co., Tsukuba, Ibaraki 300-2635, Japan*

In search for effective HIV-1 transcription inhibitors, more than 75,000 compounds have been evaluated for their inhibitory effects on Tat-induced HIV-1 long terminal repeat (LTR)-driven gene expression. Consequently, we have recently found that EM2487, a small molecule (MW: 829) substance produced from a *Streptomyces* species, is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells. Its 50% effective concentration (EC_{50}) was 0.27 μM in acutely infected peripheral blood mononuclear cells (PBMCs), while the 50% cytotoxic concentration (CC_{50}) for mock-infected PBMCs was 13.3 μM. EM2487 proved inhibitory to a variety of HIV-1 strains and HIV-2 in different cell lines (MOLT-4 and MT-4). The compound could suppress tumor necrosis factor (TNF)-α-induced HIV-1 production in latently infected cells (OM-10.1 and ACH-2) and constitutive viral production in chronically infected cells (MOLT-4/III_B and U937/III_B) without affecting cell proliferation. The EC_{50} and CC_{50} of EM2487 in OM-10.1 cells were 0.075 and 12.5 μM, respectively. EM2487 did not affect early events of the HIV-1 replication cycle, as determined by HIV-1 proviral DNA synthesis in acutely infected cells. However, the compound could prevent viral mRNA synthesis in chronically infected cells, indicating that HIV-1 inhibition occurs at the transcriptinal level. Furthermore, EM2487 did not inhibit TNF-α-induced but did inhibit Tat-induced HIV-1 LTR-driven gene expression in CEM cells, irrespective of the presence or absence of the nuclear factor κB (NF-κB) binding sites in the HIV-1 LTR. These results indicate that EM2487 is a selective inhibitor of Tat-induced transactivation.

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Indinavir Enhances Retinoic Acid Signaling: Nelfinavir, Saquinavir, and Ritonavir Inhibit Retinoid Effects *in vitro*.

E. S. FURFINE *, J. WEIEL, M. PAULIK, J. LEHMANN, G. KOSZALKA, AND J. LENHARD.

GlaxoWellcome Inc., Research Triangle Park, NC, USA

Treatment of patients with HIV protease inhibitors (PIs) or all-*trans* retinoic acid (ATRA) in some cases causes hypertriglyceridemia and hypercholesterolemia. Since PIs and ATRA may cause these changes by affecting similar molecular mechanisms, we tested the effects of PIs on retinoid signaling *in vitro*. C3H10T1/2 mesenchymal stem cells were cultured in the presence of various PIs and/or synthetic retinoids and cell-associated alkaline phosphatase (ALP) activity was measured. Basal ALP activity remained unchanged in the presence of PIs and increased in the presence of retinoic acid receptor (RAR) agonists (*e.g.*, ATRA or CH55). When used in combination with ATRA the PIs, nelfinavir, saquinavir, and ritonavir inhibited ALP activity. Amprenavir in combination with ATRA had no effect on ALP activity. However, indinavir (EC50 = 8 μM) in combination with ATRA increased ALP. The RAR-antagonist, AGN 193109, blocked the synergistic effects of indinavir and ATRA on stimulation of ALP activity. Indinavir did not potentiate ALP activity in the presence of CH55 or the RXR agonist, LGD1069. Although CH55 activates RAR, CH55 does not bind to cytosolic RA-binding protein (CRABP). Thus, one explanation for indinavir potentiating the effects of ATRA, but not CH55, is that indinavir increases displacement of ATRA from CRABP, resulting in activation of RAR and ALP. These data support the hypothesis that the side effects associated with PI therapy involves altered retinoid signaling.

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Virological, Biochemical, and Pharmacological Properties of Racemic FTC (Racivir). Schinazi, R.F.,[1]* Cretton-Scott, E.,[2] Liberman, I.,[1] Ussery, M.,[3] Liotta, D.C.[1] and Sommadossi, J.-P.[2] Emory University/VA Medical Center, Decatur, GA,[1] University of Alabama, Birmingham, AL, and FDA, Rockville, MD.[3]

The racemates of both FTC [Racivir, RCV, (±)-FTC] and BCH-189 are effective inhibitors of HIV and hepatitis B virus (HBV). DNA sequence analysis of the RT gene amplified from (+)-BCH-189-resistant viruses identified a single mutation at codon 215 from T (ACC) to Y (TAC). (+)-FTC selected in primary lymphocytes for a transient 215Y mutation. Since (+)-BCH-189 is toxic in bone marrow cells and affects mitochondrial synthesis, its racemate cannot be considered as a viable antiviral agent. However, like (-)-FTC, (+)-FTC and RCV are effective antiviral agents with no apparent *in vitro* toxicity. Since bulk manufacturing of RCV is much easier than (-)-FTC or 3TC, it was selected as a potential preclinical candidate. RCV was found to prevent the development of the mutation at codon 215, but not at 184. It is readily phosphorylated to its triphosphate form (RCV-TP) which selectively inhibits HIV RT. In human lymphocytes, reduced levels of diphosphocholine metabolite are detected compared to (+)-FTC. RCV is partially deaminated to the inactive non-toxic (+)-FTU in rhesus monkeys, rhesus monkey blood or monkey hepatocytes, whereas limited deamination (≤ 13%) to (+)-FTU occurred in human blood (3 hr at 37°C) or human liver cytosol. This suggests major differences between the two mammalian isoforms of the enzyme cytidine deaminase. RCV was as effective as (-)-FTC in an HIV-1 PBMC SCID mouse model; with undetectable virus in the peritoneum and lymph nodes at an intraperitoneal BID dose of 60 mg/kg/d for six day. These data support further development of RCV as an antiviral agent for HIV infections (Research funded in part by the VA and NIH).

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The Presence of the M184V RT Mutation is not Sufficient to Diminish Response to ABC/3TC/ZDV in Therapy Experienced Children (CNA3006) S. Danehower[1], C. Gilbert[1], R. Lanier[1] and Marty St. Clair[1] [1]GlaxoWellcome, Inc., RTP, NC

The HIV-1 reverse transcriptase (RT) gene mutations observed *in vitro* following virus passage in abacavir (ABC,Ziagen,1592) include K65R, L74V, Y115F and M184V. Combinations of these mutations are required for > 4 fold decrease in susceptibility to ABC; whereas the presence of the M184V alone is associated with only a 2-3 fold decrease in susceptibility. We examined clinical isolates from nucleoside experienced children enrolled in CNA3006, a study designed to evaluate the efficacy of ABC in combination with 3TC/ZDV vs. 3TC/ZDV to determine if baseline (BL) M184V was associated with a diminished response. A subset of subjects were identified from each treatment group that harbored isolates with the M184V mutation at BL with no other RT mutations associated with NRTI resistance (ABC/3TC/ZDV n=17, 3TC/ZDV n=16). Eleven subjects receiving ABC/3TC/ZDV and 21 subjects receiving 3TC/ZDV had wild-type virus at BL. A comparison of the week 16 HIV-1 RNA response revealed no significant treatment difference for ABC/3TC/ZDV between subject isolates harboring M184V and those harboring wild-type virus at BL. Fifty-nine percent (10/17) of subjects with M184V containing virus in the ABC/3TC/ZDV group had a decrease in HIV-1 RNA of $\geq$1.0 $\log_{10}$ copies/mL or < lower limit (LL=2.6 $\log_{10}$ copies/mL) using the Roche Amplicor Assay; as compared to 60% (6/10) with wild-type virus. In addition, development of M184V alone following ABC/3TC/ZDV therapy at 16 weeks was not associated with a diminished HIV-1 RNA response; fifty-three percent (10/19) had $\geq$1.0 or LL with M184V as compared to twenty-six percent (7/27) with wild-type virus. The presence of M184V either at baseline or emerging on-therapy was not sufficient to diminish plasma HIV-1 RNA response to ABC/3TC/ZDV as compared to wild-type virus through 16 weeks of therapy in nucleoside experienced children.

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Intracellular dATP Pools in AIDS Patients Receiving Hydroxyurea and ddI. M.A. Ussery, H. Zhang and [1]F.M. Hamzeh, FDA, Rockville, MD, USA and [1]Johns Hopkins School of Medicine, Baltimore, MD, USA.

Hydroxyurea (HU) has shown good clinical activity in the treatment of AIDS when given in combination with nucleoside therapy. *In vitro* experiments indicate that the ribonucleotide reductase inhibitor acts by reducing the intracellular pool levels of the normal nucleoside triphosphates that compete with the active antiviral drug metabolite, e.g. dATP levels that compete with ddATP in the case of ddI therapy. This reduction in intracellular pools has not been demonstrated in clinical samples from HU treated patients. In this study we have examined dATP pool levels in patients receiving HU therapy and compared them to control patients. Samples were obtained prior to (baseline) and at weeks two and eight of HU therapy. Intracellular levels of dATP have been measured in 407 patient samples by a validated analytical method that has been previously presented. Briefly, the method involves derivatization to produce a fluorescent etheno-dATP molecule that is then quantitated after HPLC separation. This method also allows quantitation of the mono- and diphosphate pools. We hypothesize that abnormally high ratios of mono- and diphosphates to the triphosphate analyte may indicate samples that were degraded by a delay between sample collection and extraction. While we have not yet unblinded the study to determine who was receiving HU therapy, a number of patients (44/87) show a significant drop in dATP levels at week 2 which is sustained in the week 8 samples. An additional sensitive and nonradioactive method (PODER) has been developed to measure the ddATP levels in the same samples, so that both competing pools can be measured simultaneously. Longer studies are needed to determine if HU-induced reductions in intracellular nucleotide pools are sustained past 8 weeks of therapy.

Poster Session I: Retrovirus, Hepadnavirus, and Other Infections

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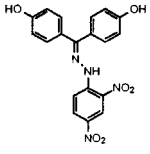
HETEROCYCLIC HYDRAZONES, A NEW CLASS OF HIV REVERSE TRANSCRIPTASE INHIBITORS : SYNTHESIS AND BIOLOGICAL ACTIVITY

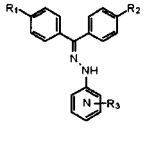
G. Pürstinger[#], M. Witvrouw[$], C. Pannecouque[$], J. Balzarini[$], E. De Clercq[$], J. Easmon[#] and G. Heinisch[#]

[$]Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium, [#]Institut für Pharmazeutische Chemie, Universität Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria

A-007 and related compounds have been described as anti-estrogens. Furthermore, anti-HIV activity is attributed to these compounds[1].



A-007



A

We have synthesized a group of analogues (**A**), in which on one hand the 2,4-dinitrophenyl moiety was replaced by π-electron deficient heterocycles and/or on the other hand the substitution pattern of the benzophenone part was varied. In the case of asymmetric benzophenones E/Z-mixtures of the corresponding hydrazones were obtained, which were separated chromatographically. The stereochemistry was assigned by means of NMR-spectroscopy. These compounds inhibit HIV replication with a selectivity index up to 60. Moreover, the mode of action of these compounds was investigated. The compounds of general formula **A** represent a novel class of non-nucleoside reverse transcriptase inhibitors (NNRTIs).

[1] L.R. Morgan, EP 0 187 039 A1 (1986); Chem. Abstr. 105: P152713

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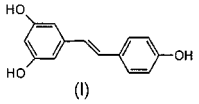
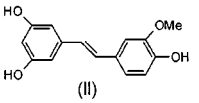
Synthesis and Anti-HIV activity of Natural products ------ Resveratrol(I) and Isorhapotigenin(II)

Lin Wang Ya-bin Feng Zhi-zhong Zhao Xing-Quan Zhang[1] Hong-Shan Chen[1] Institute of Materia Medica, [1]Institute of Medicinal Biotech-nology, Chinese Academy of Medical Sciences. Peking Union Medical College, Beijing 100050

Resveratrol(I) (E)-3,5,4'-trihydroxy stilbene, and Isorhapotigenin(II) (E)-3,5,4'-trihydroxy-3'-methoxy-stilbene are two natural products with many biological activities . Lin *et al* first isolated them from *G.parvifolium*, a traditional Chinese herb which has been used to treat rheumatoid arthritis, ulcer bleeding and bronchitis. We considered that certain polyphenolic nature products showed anti-human immunodeficiency virus (HIV-1) activity such as Tannins, Flavonoides isolated from chinese medicinal herbs. In an attempt to obtain enough samples (I) and (II) for further anti-HIV activity tests, we have developed two synthetic approaches of I and II starting from 3,5-dihydroxybenzoic acid , by eight and seven step reaction dequence,respectively. The synthetic products I and II were characterized by IR,MS, ^{1}H-NMR in comparison with the corresponding natural products. The preliminary anti-HIV test showed that the synthetic product (I) had anti-HIV activity in H_9 cell with an IC_{50} of 9.79 u M and the Therapeutic index (TI, TC_{50} / IC_{50}) was 34. We believe that Chinese herbs are a rich source of useful materials for the treatment of AIDS virus infection.



(I)



(II)

***This work is supported by the National Natural Science Foundation of China.**

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The Activity of Mg^{2+} and Poly r(A-U) against HIV-1

M. R. Nassiri[1], J.M. Jamison[2], J. Gilloteaux[3], C.-c. Tsai[4] and J. L. Summers[2]

Departments of Pharmacology/Virology[1] and Anatomy[3], Lake Erie Coll. of Osteopathic Medicine, 1858 W. Grandview Blvd, Erie, PA. 16509, Department of Urology[2], Summa Health System/N.E.Ohio Univ. Coll.of Medicine, St. Rt. 44, Rootstown, OH 44272, and Department of Chemistry[4], Kent State Univ, Kent, OH 44242, U.S.A.

Successful antiviral therapy with nucleoside analogs has been hampered by their toxicity and by viral resistance. Polyribonucleotide analogs are novel HIV inhibitors whose antiviral activity has not been fully explored. In this study polyribonucleotides alone and in combination with Mg^{2+} were tested for their antiviral activity against HIV and VSV. When Mg^{2+} was pre-incubated with poly r(A-U) or poly r(G-C) and tested in a human foreskin vesicular stomatitis bioassay, the 50% effective doses decreased up to 10-fold. Polydeoxyribonucleotides (PDN) alone, Mg^{2+} alone and the Mg^{2+}/PDN combinations were not efficacious antiviral agents. The enhanced antiviral activity was not due to increased interferon production or direct viral inactivation and no host cell toxicity was observed. A p24 ELISA assay system demonstrated that Mg^{2+} and the Mg^{2+}/poly r(A-U) combination exhibit potent activity against HIV-1 3B infected peripheral blood mononuclear cells. Since phase contrast micrographs indicate the Mg^{2+}/poly r(A-U) combinations localize in the nucleoli and chromatin of the host cells, modulation of nuclear (nucleolar) processes may be responsible for the enhanced antiviral activity. Because the Mg^{2+}/polyribonucleotide combinations are efficacious against both viruses, the clinical efficacy of this novel combination should be explored further.

Funded by Kent State/Summa Health Research Foundation.

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Salivary Glycoproteins Specifically Inhibit HIV-1 Infectivity.

D. Malamud, CA Davis, and WR Abrams, Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia Pa 19104

Incubation of HIV-1 with human saliva inhibits infectivity *in vitro*. If this phenomenon occurred *in vivo*, it could explain the absence of oral transmission of HIV. Our studies were designed to identify the active salivary molecules and elucidate their mechanism of action. Dialyzed, lyophilized submandibular saliva, collected from HIV-seronegative donors, was incubated with virus. Infectivity of treated virus was analyzed in PBMCs (p24 ELISA), Sup-T1 cells (syncytia formation), or HeLa-CD4-βgal cells (MAGI assay), as compared to cells incubated with virus only. We have isolated a fraction from human submandibular saliva, utilizing anion exchange chromatography, that demonstrates potent anti-viral activity. The active fraction contains two high molecular weight sialyated glycoproteins; salivary agglutinin (MW360,00) and the mucin MG2 (MW 180,000). The two glycoproteins were purified from human saliva and tested against representative T-tropic, M-tropic, and dual-tropic strains of HIV-1. Both glycoproteins demonstrated potent, dose-dependent inhibition of HIV-1 infectivity. The interaction of these salivary glycoproteins with gp120 was studied using using biosensor technology. The active fractions isolated from submandibular saliva bound to immobilized gp120 with affinities similar to sCD4 binding to gp120. Purified salivary agglutinin demonstrated a complex binding pattern with two binding constants, reflecting high and low affinity sites. These results suggest that the salivary glycoproteins specifically bind to gp120 with high affinity, and that this interaction is involved in the inhibition of HIV-1 infectivity.

Supported by NIH grant DE 12830.

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Inhibitory effects of cytochalasin D and demecolcine on human immunodeficiency virus type I replication in vitro

Khakhulina T.V.,[1] Bourchteine M.E.,[2] Vorkunova G.K.,[1] Bukrinskaya A.G.[1]

[1]D.I.Ivanovsky Institute of Virology, [2]AIDS Prevention Center «ANTIVICH», Moscow, Russia.

Studying host cell cytoskeleton involvement into HIV-I life cycle we investigated the influence on virus replication of the cytochalasin D (CCD), an inhibitor of actin polymerization, and demecolcine, the agent which causes perinuclear aggregation of intermediate filaments. HIV-I infected MT-4 cells were fractionated into cytosolic, nuclear, cytoskeletal and membrane fractions. CCD treatment reduced the amount of nuclear and cytoskeletal Gag MA protein which represents a marker for viral reverse transcription complex. Demecolcine and CCD inhibited viral infectious activity in single cycle infectivity assay and reduced the p24 accumulation in supernatants as was shown by ELISA. However, unlike CCD which strongly inhibited viral infectivity only when the cells were pretreated with the agent, demecolcine diminished viral infectivity even when added 6 hours and later on after infection. So far, actin network and intermediate filaments are involved into different steps of HIV-I replication. Studying virus-cytoskeleton interaction could give an impetus to the search of new targets for antiviral therapy of AIDS.

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NEWLY DEVELOPED POLYANIONIC DERIVATIVE OF NORBORNENE INHIBITS HIV-1 REPLICATION.

M. E. Bourchteine[1], A. V. Serbin[2], T. V. Khakhulina[3], L. I. Kasyan[2], A. O. Kasyan[2], A. G. Boukrinskaya[3]

[1]AIDS Prevention Center «ANTIVICH», [2] Health Research & Development Foundation, [3]Ivanovsky Virology Institute, Moscow, Russia.

Objectives: To study the mechanism of inhibition of the HIV-1 replication by newly developed polyanionic derivative of nonbornene.

Methods: The drug was added to HIV-1 infected MT 4 cells, with the virus, 1 hour and 2 hours after infection, or the MT-4 cells were pretreated with the drug 2 hours before infection The effect of the drug was shown by immunoblotting of cell lysates. The drug was also tested in HIV-1 infected Hela CD 4+ - β - galactosidase cells («Magi» cells). The effect of the drug was shown by measuring of OD. The absence of cytotoxic effect was shown by MTT test for estimating cytotoxic dose (CTD_{50}).

Results: The strong HIV-1 inhibition was shown only when the drug was added with the virus, or when the cells were pretreated with the drug .
The weak inhibition was shown when the drug was added 1 hour after infection. The transport of *gag* matrix protein into the nuclei was blocked. When the drug was added later on the inhibition was not detected. The drug does not impair the virus structure and does not impair adsorption.

Conclusions: The compound affects some early steps of virus replication following adsorption such as uncoating and/or nuclear transport of viral complex.

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NORBORNENE CONTAINING ANTIVIRALS: SYNTHESIS AND EVALUATION OF NEW POLYANIONIC DERIVATIVES.

A.V. Serbin[1], L.I. Kasyan[1], M.E. Bourchteine[2], A.G. Bukrinskaya[2]

[1] Biomodulators & Drug Research Department of Health Research & Development Foundation; [2] Ivanovsky Virology Institute, Moscow, Russia.

Objectives. To develop the approach for amplification of a polycyclic hydrocarbons antiviral potential by means of cooperation of their hydrophobic-targeting selectivity with hydrophilic-sensitive activity of the special polyanionic carriers. Previously studied on polyanionic derivatives of adamantane (**AMANT**), this approach is used for creation of new norbornene containing membranotropic antivirals (**NAVs**).

Methods. NAV were synthesized as described earlier for **AMANT** and has similar tri-component structure: ***Norbornene - Spacer - Polyanionic carrier*** (MW =<15KD). Cytotoxic effect was assayed on MT-4 cells by MTT test. Antiviral activity was evaluated against **HIV-1** (strains: X794 LAI, H9/IIIB and MFA) by syncytium formation assay, detection of the viral proteins p24 and p55 on MT-4 cells as well as by determination of β-galactosidase production after hydrolysis of X-gal on "Magi" cells.

Results. Unlike norbornene, the polyanionic-modified derivatives with 5-15 wt.% of norbornene are well soluble in water-based media. The new substances have low cytotoxicity (CC_{50} ~800-900 μg/ml), and **NAV** with optimal hydrophobic-hydrophilic balance/molecular design manifest the strong inhibition of **HIV-1** infection. The best results were observed when the antivirals were added to cells simultaneously with virus or the cells were pretreated with the drugs. **NAV** inhibited some early steps of virus replication. The most promising **NAV** (**As-504**) exhibits 50% inhibition of **HIV-1** (X794 LAI) at low concentrations: IC_{50}=0.08 μg/ml (~10 nM) [D.D.Richman's measurement], which corresponds to high *selectivity index* = CC_{50}/IC_{50} =**10 000**.

Conclusions: similar to recently investigated **AMANT**, the norbornene containing antivirals possess low cytotoxicity and strong inhibition of **HIV-1** replication. Moreover, the *newly developed norbornene derived agents* are *more effective* **HIV-1** *inhibitors* than corresponding adamantane analogues.

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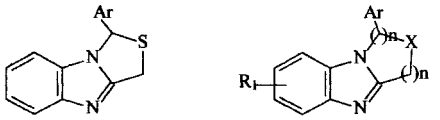
HIV-1 RT INHIBITORS CONTAINING A CYCLOFUNCTIONALIZED BENZIMIDAZOLE SYSTEM

A. Chimirri,[1] E. De Clercq,[2] S. Grasso,[1] A.M. Monforte,[1] P. Monforte,[1] C. Pannecouque,[2] A. Rao,[1] M. Witvrouw,[2] M. Zappalà[1]

[1]Dipartimento Farmaco-Chimico, Università di Messina, 98168 Messina, Italy
[2]Rega Institute for Medical Research, B-3000, Leuven, Belgium

A large number of drugs currently marketed for treating HIV-1 infections target the reverse transcriptase (RT) which is an essential enzyme that operates in the early phase of the viral replication cycle. HIV-1 RT inhibitors fall into two general classes: nucleoside analogues (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). Our previous publications (1a-c) reported the synthesis and biological results of a series of 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles **I** (TBZs) which proved to be potent and selective HIV-1 inhibitors. TBZs are active against a panel of biologically diverse strains of HIV-1 and, in combination with AZT and ddI, synergistically inhibit HIV-1-induced cell killing. The key structural requirements for an efficient enzyme inhibition by this class of compounds are analogous to other NNRTIs such as TIBO and nevirapine. They have the capability of assuming a butterfly-like conformation and the possibility of accommodating suitable lipophilic and electronic groups (2a-b). Furthermore, comparative molecular modeling studies and dynamic docking experiments suggest the feasibility to improve the anti-HIV-1 activity by introducing some substituents of different size and shape and/or modifying the tricyclic system. The synthesis and structure-function relationship of new benzimidazole TBZ analogues (**II**) will be reported. This will enable us to characterize the main intermolecular interactions involved in the RT inhibition process.



I **II**

1. a) A. Chimirri et al. *Il Farmaco* **46**, 817 and 925 (1991); b) ibid. **52**, 673 (1997); c) *U.S. Patent*, 5,217,984 (1993). 2. a) A. Chimirri et al. *Antiviral Chem. Chemother.* **8**, 363 (1997); b) ibid. **9**, 431 (1998).

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HIV Protease Inhibitors Block Adipogenesis and Increase Lipolysis *in vitro*. E. S. FURFINE *, J. WEIEL, M. PAULIK, L. MILLER, O. ITTOOP, S. BLANCHARD, AND J. LENHARD. GlaxoWellcome Inc., Research Triangle Park, NC, USA

Patients receiving HIV protease inhibitor (PI) therapy often suffer from a syndrome of lipodystrophy, characterized by the loss of subcutaneous fat from the face and limbs. As the cause of this syndrome is unknown, we tested the effects of PIs on adipocyte differentiation and fat metabolism *in vitro*. C3H10T1/2 mesenchymal stem cells were cultured in the presence of various PIs under conditions that promote adipogenesis (*i.e.*, in the presence of insulin and agonists for the nuclear receptors PPARγ and RXR). The PIs, nelfinavir, saquinavir, and ritonavir, inhibited several markers for adipogenesis, including total triglyceride accumulation, lipogenesis, and expression of the adipose markers, aP2 and LPL, in these cells. Histological analysis revealed nelfinavir, saquinavir and ritonavir treatment decreased oil red O-staining of cytoplasmic fat droplets. Moreover, these three PIs increased acute lipolysis in C3H10T1/2 adipocytes. In contrast, two HIV PIs, amprenavir and indinavir, had little effect on lipolysis, lipogenesis, or expression of aP2 and LPL in C3H10T1/2 cells. Further, none of the PIs bound to the nuclear receptors RXR or PPARγ, (IC50s > 10 μM) indicating that inhibition of adipocyte differentiation was not due to antagonism of ligand binding to RXR or PPARγ. Taken together, the results suggest several, but not all, HIV-PIs block adipogenesis and stimulate fat catabolism *in vitro*. These effects may contribute to the lipodystrophy associated with PI therapy.

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Synergistic inhibition of HIV-1 replication by combination of the transcription inhibitor K-12 and other anti-retroviral agents in acutely and chronically infected cells

M. Okamoto, T. Okamoto* and M. Baba *Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520 and *Department of Molecular Genetics, Nagoya City University Medical School, Nagoya 467-0001, Japan*

The fluoroquinoline derivative K-12, 8-difluoromethoxy-1-ethyl-6-fluoro-1,4-dihydro-7-[(4-(2-methoxyphenyl)-1-piperazinyl]-4-oxoquinoline-3-carboxylic acid, has recently been identified as a potent and selective inhibitor of HIV-1 replication at the transcriptional level. The compound is inhibitory to HIV-1 replication in both acutely and chronically infected cells. In this study, we have examined several combinations of K-12 and other anti-retroviral agents for their inhibitory effects on HIV-1 replication in acutely and chronically infected cell cultures. Combinations of K-12 and the reverse transcriptase (RT) inhibitor, either zidovudine (ZDV), lamivudine (3TC), or nevirapine (NVP), synergistically inhibited HIV-1 replication in acutely infected MT-4 cells. Combination of K-12 and the protease inhibitor nelfinavir (NFV) also exhibited synergistic inhibition of HIV-1 replication, whereas the synergism of this combination was weaker than that of the combinations with the RT inhibitors. Synergism of the combinations was also observed in acutely infected peripheral blood mononuclear cells. K-12 did not enhance the cytotoxicity of RT and protease inhibitors. Combination of K-12 and cepharanthine (CEP), a nuclear factor κB (NF-κB) inhibitor, synergistically inhibited HIV-1 production in tumor necrosis α-stimulated U1 cells, a promonocytic cell line latently infected with the virus. In contrast, additive inhibition was observed for combination of K-12 and NFV. These results indicate that the combinations of K-12 and clinically available anti-retrovirals may have potential as chemotherapeutic modalities for the treatment of HIV-1 infection.

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Synergism studies between efavirenz (SUSTIVA™, DMP266) and nucleoside or non-nucleoside inhibitors of the HIV-1 RT. R.W. King, C.D. Reid, R.M. Klabe, S. Garber, and S.K. Erickson-Viitanen. DuPont Pharmaceuticals Co., Wilmington, DE, USA, 19880-0336.

Efavirenz (DMP 266; SUSTIVA™), a non-nucleoside inhibitor of the HIV reverse transcriptase (RT), is a potent inhibitor of HIV replication [EC_{50} = 3 nM in yield reduction assays using MT-2 cells infected with HIV-1 (RF)]. It recently has been approved by the U.S. Food and Drug Administration for use in combination with other anti-HIV drugs for treating individuals infected with HIV. We have carried out *in vitro* combination studies using efavirenz in combination with the nucleoside RT inhibitor AZT or with one of several non-nucleoside RT inhibitors (i.e. nevirapine, DPC 961, or DPC 963). These assays were performed in both an enzymatic RT assay in which RNA-dependent DNA polymerase HIV-1 RT activity was measured by the incorporation of ^{3}H-TMP into the newly synthesized DNA strand; and a cell-based assay using yield reduction in MT-2 cells infected with HIV-1 (RF) as the endpoint. To determine if the drug combinations acted synergistically, the data was analyzed using isobolograms and Combination Index plots. We found that when efavirenz and AZT were used in combination in either the enzyme or yield reduction assays, these two drugs acted synergistically to inhibit RT activity and virus replication, respectively. The enzyme assay data agree with what has been previously reported by another group (Young, et al., Antimicrob. Agents Chemother., 39:2602-2605). In contrast, preliminary data with efavirenz in combination with a closely related compound, DPC963 showed that although these compounds acted additively in inhibiting RT activity in the enzyme assay, they acted antagonistically in inhibiting HIV-1 replication, suggesting that other factors independent of enzyme:inhibitor interaction may influence the potency of two compounds when used in combination in cell culture.

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Anti-HIV activity, drug combination, and resistance studies of dOTC (BCH-10652)

J. Bedard[1], D.L. Taylor[2], L.A. Kelly[2], L.A. Wood[2], A.S. Tyms[2], J.M. McCune[3], C.A. Stoddart[3], and R.F. Rando[1].
[1]BioChem Pharma Inc., Laval, Canada, [2]MRC Collaborative Center, Mill Hill, London, U.K., [3]Antiviral Drug Research Division, Gladstone Institute of Virology and Immunology, UCSF, San Francisco, CA, USA.

dOTC (BCH-10652) is a 2,4-disubstituted 1,3-oxathiolane cytosine nucleoside currently in phase I/II clinical trials. Antiviral efficacy studies of dOTC and its enantiomers BCH-10618 and BCH-10619 have demonstrated that these compounds are associated with a potent and selective activity against wild-type clinical isolates of HIV-1 cultured in PBMCs with IC_{50}s ranging from 0.1 to 4.8 μM. The potency against viral isolates resistant to 3TC® or to 3TC® and AZT was found to remain relatively unchanged for dOTC and BCH-10618 with approximately 2-fold increase in the mean IC_{50} values. *In vivo* efficacy studies were performed using SCID-hu Thy/Liv mice infected with HIV-1 isolate NL4-3. In Thy/Liv implants, we observed a dose-dependent reduction of p24 antigen production and HIV-1 RNA load in mice treated by oral gavage with dOTC. For example, at day 12 post-infection, a 55, 78, and 94 % reduction in the level of p24 was observed at doses of 30, 90, and 200 mg/kg/day respectively of dOTC. *In vitro* drug combination studies using MT4 cells infected with HIV_{RF} strain demonstrated an additive interaction of dOTC with compounds currently used in anti-HIV therapy such as 3TC®, AZT, ddI, nevirapine, indinavir, ritonavir, and saquinavir. Experiments aimed at selecting dOTC-resistant HIV-1 strains in cell culture revealed that resistance is slow to develop when compared to 3TC®. A 1000-fold increase in the IC_{50} to 3TC® was obtained for HIV_{RF} strain cultured in C1866 cells after 8 serial passages in the presence of increasing concentrations of drug, whereas for dOTC, >12 passages were required for selection of resistant viruses. Mutations at RT codons 65 and 184 were observed in the dOTC resistant isolates. The anti-HIV properties and resistance profile of dOTC help support the clinical development of this compound.

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VX-497, a novel IMPDH inhibitor, potentiates the antiviral activity of ddI and ddG *in vitro*. A.D. Kwong and R.A. Byrn, Vertex Pharmaceuticals Inc., 130 Waverly St., Cambridge MA 02139 USA.

IMPDH inhibitors have been shown to potentiate the antiviral effects of purine nucleoside analogs, including inhibitors of HIV and herpes virus infection. This is thought to occur through depletion of the dGTP pools, and elevation of IMP pools, which results in a more efficient phosphorylation of the nucleoside analog and an enhancement of antiviral activity. In this study we show that the novel IMPDH inhibitor VX-497 potentiates the activity of ddI or ddG against HIV-1 in a MT-4 cell CPE-based assay. Using the rapid screening method of St Clair et al (J AIDS and Human Retrovir 10[S1]: S24-S27); serial dilutions of one anti-retroviral agent were prepared, and tested for activity in the presence or absence of a constant level of a second agent. We first evaluated ddI as the variable anti-HIV agent, and performed antiviral assays in the presence and absence of 10 μM ribavirin. The addition of ribavirin shifted the dose response curve for ddI, resulting in a decrease in the IC_{50} for ddI from 9.7 μM to 0.8 μM. When similar experiments were performed using ddI in the absence and presence of 0.8 μM of the IMPDH inhibitor VX-497, the IC_{50} for ddI was shifted from 9.7μM to 2.9μM. This means that VX-497 potentiates the antiviral activity of ddI by 3.3-fold in this assay system. This potentiation of ddI activity was eliminated when VX-497 was combined with 50 μM guanosine and 8-aminoguanosine, consistent with this effect being the result of IMPDH inhibition. Similar results were obtained when ddG titrations were performed in the absence or presence of VX-497, resulting in a shift of the IC_{50} for ddG from 4.5 μM to 1.3 μM. The 3.5-fold potentiation of ddG activity was again eliminated when VX-497 was combined with 50 μM guanosine and 8-aminoguanosine. Together, these results suggest that VX-497 has the capacity to potentiate the activity of purine nucleoside antiviral inhibitors.

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The RNA Binding Domain of HIV-1 Nucleocapsid Precursor Protein p15: A Novel Anti-HIV-1 Target

D. H. Ozturk, A. Draganescu and S. Erickson-Viitanen. DuPont Pharmaceuticals Company, Wilmington, DE 19880, USA

Infectious viral particle assembly process of HIV-1 requires the timely sequential cleavage of the *gag* gene product by HIV-1 protease during virus maturation. The cleavage products include proteins p17 (matrix), p24 (capsid) and p15. The p15 protein is further processed by HIV-1 protease to yield p7 (nucleocapsid), p6 and p1 polypeptides. The timely and efficient cleavage of p15 protein requires the presence of viral RNA. A 24mer RNA oligo derived from the viral RNA sequence and its 21mer antisense DNA oligo was shown to be also sufficient for complete cleavage of p15. Photoaffinity labeling was used to determine the amino acids involved in interacting with the 21mer DNA oligo. 5-iodouracil or 5-iodocytidine substituted 21mer DNA oligos were used for photo-crosslinking experiments. Protein•oligo complex was irradiated by a HeCd laser source at 325 nm. Initial results of photoaffinity labeling experiments indicated that Cys-28, Cys-18, Met-46 and Glu-42 are located in the RNA binding domain of p15. To determine the structural requirements and sequence specific interactions of the RNA or DNA oligos with p15, DNA footprinting and base deletions or sequence altered oligo derivatives were utilized in binding and cleavage assays. The complete cleavage of p15 into p7, p1 and p6 is dependent on stem-loop structure and the sequence of the oligo present in the cleavage assays. Based on the binding studies and the cleavage assays, the stem region and guanosine at position 14 in 21mer DNA oligo and cytosine at position 8 in the 24mer RNA oligo appear to be important for binding. DNA footprinting studies suggested that the 4' hydroxyl groups of the sugar backbone of thymidines in the loop region of 21mer DNA oligo interact with p15. The RNA-dependent processing of p15 and the sequence specificity of the RNA binding site implicate a new therapeutic target in HIV-1. Chemical agents that can impair the RNA binding and thus interfere with timely processing of p15 by HIV-1 protease may disrupt infectious viral particle formation during the life cycle of HIV-1.

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Cytotoxic and anti-HIV-1 effects of amphiphilic heterodinucleoside phosphates containing AZT and ddC in H-9 cells with induced AZT resistance

B. Gröschel[1], R. Zehner[2], H. Schott[3], R. A. Schwendener[4], J. Cinatl[1,5], J. Cinatl Jr.[1], H. W. Doerr[1] [1]Institute of Medical Virology, Johann Wolfgang Goethe University, Sandhofstr. 2-4, 60528 Frankfurt/M., Germany; [2]Center of Legal Medicine, Johann Wolfgang Goethe University, Frankfurt/M., Germany; [3]Institute of Organic Chemistry, University of Tübingen, Germany; [4]Department of Pathology, Division of Cancer Research, University Hospital Zürich, Switzerland; [5]Center of Pediatrics, Department of Hematology and Oncology, Johann Wolfgang Goethe University Frankfurt/M., Germany

The most important reason for the waning efficiency of antiretroviral agents is the development of resistance mechanisms. Resistant virus mutants with minor susceptibility against chemotherapeutic agents rapidly emerge after long-term treatment of HIV infected patients. In addition, different cellular resistance mechanisms including decreased intracellular activation of chemotherapeutic agents due to altered activity of nucleoside kinases may contribute to decreased efficiency of 2',3'-dideoxynucleoside reverse transcriptase inhibitors. We have selected an AZT- and d4T-resistant T-lymphoid cell line (H-9^{r}AZT^{250}) by continuous cultivation of the cells in medium containing increasing concentration of AZT. H9^{r}AZT^{250} cells were used to study the mechanisms of cellular resistance and to test the efficiency of monophosphorylated substances in these resistant cells. Thymidine kinase (TK) mRNA level was decreased in H9^{r}AZT^{250} cells in comparison to parental cells, which has been characterized by RT-PCR. Furthermore, cytotoxicity and antiretroviral activity of several amphiphilic heterodinucleoside phosphates containing AZT and ddC were tested for their ability to overcome AZT induced cellular resistance mechanisms in H9^{r}AZT^{250} cells. Cytotoxicity was tested by the MTT assay and anti-HIV-1 activity of the drugs was determined by measurement of HIV-1-p24-antigen in cell culture supernatant. The ddC and AZT containing heterodimers showed comparable cytotoxic and antiretroviral effects in AZT resistant and parental cells. These results demonstrated that heterodinucleoside phosphates release the nucleoside monophosphate form of AZT, which was able to overcome cellular resistance mechanisms, such as TK deficiency in H-9^{r}AZT^{250} cells.

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Didox, a Novel Ribonucleotide Reductase Inhibitor, is More Active Than Hydroxyurea in HIV-infected HuPBMC SCID Mice. M.A. Ussery, O.L. Wood, D.D. Broud, M.A. Bacho, S.C. Kunder, S.F. Vona, C.J. Nielsen, and H.L. Elford[1], FDA, Rockville, MD, USA and [1]Molecules for Health, Richmond, VA, USA.

Recently Hydroxyurea (HU) has shown good clinical activity in the treatment of AIDS when given in combination with nucleoside therapy. We tested Didox, a more potent ribonucleotide reductase inhibitor, to determine if it might be more active *in vivo* than HU. Preliminary dose ranging experiments were performed in the Rauscher murine leukemia model and were used to estimate optimal HU and Didox concentrations for activity and toxicity. These optimal concentrations of 600 mg/kg HU and 450 mg/kg Didox were used to treat HIV-infected SCID mice reconstituted with human PBMC. Infectious HIV recovery was measured in blood cells, splenocytes, lymph nodes and peritoneal cells by quantitative coculture. Intracellular viral RNA copy number (viral load) was quantitated by the NASBA assay. The ability of treatment to protect human CD4 lymphocytes from virus-induced cytolysis was measured by FACS analysis. HU and Didox were tested alone and in combination with ddI, with i.p. drug therapy beginning 24 hr before HIV infection. Didox showed significantly more activity than HU when given as monotherapy. For example, in peritoneal cells Didox reduced viral titers from 4.8×10^3 $TCID_{50}/ 10^6$ cells to 2.2×10^1 $TCID_{50}/ 10^6$ cells while HU treatment reduced the titers to 2.7×10^3 $TCID_{50}/ 10^6$ cells. Combination regimens were highly effective at inhibiting HIV replication and could not be differentiated in this experiment. Additional combination experiments are indicated to differentiate between the two regimens. Didox showed promising antiviral activity in the SCID model and warrants clinical evaluation.

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GW420867X, a New Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) - Initial Phase I evaluation.

W. Prince, K. Moore , L. Cass, N. Dallow, A. Jones, J-P. Kleim, P. Mutch and M. St. Clair[1] GlaxoWellcome R&D, Greenford, UK & Research Triangle Park, NC, USA[1].

GW420867X is a new quinoxaline derivative and NNRTI that is effective at inhibiting HIV-1 reverse transcriptase (RT) by an allosteric mechanism. GW420867X is inactive against the HIV-2 RT. Potent inhibition in the nanomolar range by GW420867X of laboratory and clinical HIV-1 isolates has been demonstrated in different cell types. When foetal calf serum was replaced by human serum the inhibitory effect of GW420867 on replication of HIV-1 HXB2 in MT4 cells was retained (IC_{50} =11nM). Importantly the addition of human serum had less effect in reducing the IC_{50} than other NNRTI's tested. Pharmacokinetics, safety, and tolerability have been evaluated in 2 randomized, double-blind, placebo-controlled studies following single and repeat dose administration in healthy male volunteers. Single oral doses have been studied over the range 10mg to 1200mg and repeat oral doses of 10, 50, 100 and 200mg QD have been given for 14 days. GW420867X was readily absorbed although T_{max} for the 1200mg was delayed. Following repeat dose administration the median T_{max} was approximately 1-2h and steady-state plasma concentrations were generally achieved in 5-7d. Single dose exposure was not predictive of repeat dose exposure since GW420867X steady-state exposure (AUC_{24}) was generally 20-30% less than expected based on concentrations measured after the first dose for the 50, 100 and 200mg dose groups. However, mean accumulation ratios for C_{max} and AUC were generally 1.8 and 2 fold, respectively, which is consistent with the terminal elimination t½ of ~50h. GW420867X was generally well tolerated in all groups with headache the most common adverse event (AE). The incidence of AEs was greater in the 900 and 1200mg single dose groups. Because of the favorable pharmacokinetic and safety profile GW420867X has been selected as a candidate for clinical development and is being evaluated in subjects infected with HIV-1. The steady state levels seen in these Phase I studies and the relatively low protein binding of ~90% indicate that GW420867 should be an effective addition to currently available antiretrovirals for the treatment of HIV-1 infection.

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VALIDATION OF SURROGATE MARKERS FOR USE IN CLINICAL TRIALS FOR HIV/AIDS AND OTHER VIRAL DISASES. Jonathan Kagan, NIH, Bethesda, MD and Donna Mildvan, Beth Israel Hospital, New York, NY. Since the earliest days of the HIV/AIDS epidemic, numerous studies have addressed the apparent relationships in HIV-infected individuals between immunologic and virologic parameters and clinical disease. Relatively few laboratory measures, however, have been translated into practical application as "markers" for use in prognostics, therapeutics, vaccine development or individualized case management. Most commonly this has been due to: 1)failure to validate preliminary observations made in small cross-sectional studies; 2) an obscure relationship between the "marker" and disease pathology and/or lack of specificity for HIV disease; 3) technical barriers; 4) the complexity of the marker validation process and; 5) the potential for serious misestimates of clinical benefit when relying solely on surrogate markers. Despite an extensive literature that has enabled both the framework and the criteria necessary to define a useful surrogate, there has remained a lack of consensus among clinical and basic researchers and statisticians as to the best approach to surrogate marker validation and application. We have proposed a paradigm for the definition and validation of immunologic and virologic markers for HIV disease. We shall present the paradigm as a common platform to expedite the development of laboratory markers for application in a variety of clinical interventions and diseases. Implicit in the approach is the requirement to conduct marker validation studies in the context of interventions known to confer clinical benefit. In order to define the essential host elements that contribute to clinical benefit, candidate markers must be validated in trials of drugs whose clinical efficacy has been demonstrated. Once validated in that setting, markers can be used to evaluate new treatments. And, if novel interventions (such as immune-based therapies) prove clinically beneficial, opportunities will emerge for new marker validation studies.

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INDINAVIR AND RITONAVIR IN THE CEREBROSPINAL FLUID (CSF) OF HIV-INFECTED PATIENTS: ANTIVIRAL EFFECTS AND TRANSPORT MECHANISMS

L Ståhle, C Martin[1], J-O Svensson, A Sönnerborg[2]
Clin. Pharmacology, [1] Neurology and [2] Infectious Disease and Clin. Virology Dept. Hudding Univ. Hosp., Karolinska Inst. SE-14186 Huddinge, Sweden.

We reported recently that indinavir can be measured in the CSF of HIV-infected patients. In this study we have extended the analysis of indinavir to 32 samples in 25 patients, on triple therapy, with parallell sampling of plasma such that the variation in plasma and CSF can be followed over time. The CSF samples were also analysed for HIV-RNA levels which were compared with 36 HIV-infected untreated patients. In 4 patients on ritonavir treatment the levels in CSF have been measured in parallell with plasma samples analysed for total and unbound concentration. A pharmacokinetic analysis of indinavir data revealed an active transport from the CSF to plasma with the consequence that CSF levels were relatively stable around 200 nmol/l while plasma levels fluctuated as expected over the dose interval i.e. almost two orders of magnitude. CSF-RNA was reduced significantly in indinavir treated patients compared to untreated controls. Ritonavir treated patients also had measurable levels in CSF (28±11 nmol/l) which is lower than the unbound plasma levels (95±18, n=3) indicating that also ritonavir is actively transported out from the CSF. The total plasma ritonavir was 13.8±4.6 umol/l and CSF levels should thus be related to unbound plasma levels to be interpretable. It is concluded that indinavir and ritonvir both reach the CSF in concentrations lower, but not much so, than plasma and that, at least indinavir but possibly also ritonavir, has an effect on HIV-RNA levels in the CSF.

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HIV Gene Therapy: the Good, the Not So Good, and the Opportunities. N. Sarver, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA.

Close to 10 years ago, the notion of gene therapy (GT) as a therapeutic approach against HIV was raised. The theoretical appeal of the strategy - suppressing HIV with a therapeutic gene - has struck a chord in the research community. In the intervening years, the growing field has seen progress, setbacks, adaptations, and an increased appreciation of the complexity and intricacy of GT. GT comprises many interrelated disciplines including virology, cell biology, molecular biology, immunology, hematology, and vectorology: it is with comprehensive, carefully planned, and iterative studies that further advances are likely.

It is prudent at this juncture to (i) restate the goals of GT for HIV: what are the realistic expectations? (ii) evaluate preclinical and clinical studies: have they supported and/or reinforced the original premise of GT? (iii) review lessons learned thus far: have these been used optimally to plan subsequent studies, re-design clinical protocols, and generally advance the field? iv) re-examine GT in the context of HAART: is the opportunity of conducting GT studies in the absence of high viral burden fully appreciated? Are protocol designs and clinical endpoints in concert with current HIV clinical management?

An expanded discussion of the above and current status of on-going HIV GT protocols will be presented.

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NIH HIV/AIDS Therapeutics Discovery & Development Resources Available to Support Novel Candidates & Strategies. N. Sarver and J. Kagan, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD USA.

The Division of AIDS (DAIDS) NIAID/NIH targets significant resources for preclinical discovery and development of new and under-explored therapies for HIV/AIDS, related opportunistic diseases and topical microbicides. Discovery resources include access to chemical and biological databases and grant support for basic research in HIV pathogenesis, immunology, virology and molecular biology. Multidisciplinary consortia of academic, industry and government investigators (domestic and foreign) collaborate to advance knowledge in "gap" areas targeted for preclinical discovery and development. Once a "discovery" has been made and biologic activity confirmed, candidate therapeutics can be advanced, with DAIDS support, through preclinical evaluation (e.g. pharmacology, toxicology) to proof-of-concept clinical studies and, if warranted, into larger scale human trials. DAIDS development resources provide academic, industry and government investigators (domestic and foreign) access to *in vitro* screens (e.g. virologic, immunologic, specific pathogen) and *in vivo* models (e.g. SCID mouse, FIV cat, SIV monkey) for activity and/or efficacy. In addition, investigators may obtain assistance in analytical chemistry, chemical synthesis of GMP-grade material for clinical trials, formulations and dosage-form manufacturing. Resources are allocated in accordance with NIH AIDS research priorities. Opportunities to access these valuable resources for accelerated therapeutics development, and guidance for the submission of requests for assistance, and opportunities for collaboration will be presented.

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Inhibition of Hepatitis B Virus *in vitro* by 9-[2-methylidene-3-(phosphonomethoxy)propyl]guanine (MPMPG)

S.F.Costelloe, D.L.Taylor, P.Chambers, P.Ahmed, and A.S.Tyms. MRC Collaborative Center, 1-3 Burtonhole Lane, Mill Hill, London, U.K.

The novel acyclonucleotide derivative of guanine, 9-[2-methylidene-3-(phosphonomethoxy)propyl]guanine (MPMPG) has previously been reported to have activity against the replication of Human Immunodeficiency Virus (HIV) both *in vitro* and in the hu-PBL-SCID.Beige mouse model of infection. We now report that this compound also shows potent and selective inhibition of Hepatitis B Virus (HBV) replication *in vitro*. Anti-HBV activity was determined by treating the the HBV DNA transfected HepG2 cell line, 2.2.15, with different concentrations of compound for 10 days and measuring the levels of HBV DNA in the cell-free culture fluid by dot-blot analysis. MPMPG (mean IC_{50} 1.10μM ± 0.68μM), had slightly better activity than that of adefovir (PMEA) (mean IC_{50} 3.18μM ± 1.01μM), but was less active than 3TC (mean IC_{50} 0.047μM ± 0.022μM). Southern blot analysis of 2.2.15 cells treated with MPMPG indicated a significant reduction in HBV replicative intermediates and confirmed the antiviral effect of this compound. The potential of MPMPG for clinical progression will be discussed.

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Use of Digoxigenin-Labelled Probes for the Detection of HBV-DNA in Antiviral Evaluation Assays

C. YING[1], J. VAN PELT[2], P. YAP[2], E. DE CLERCQ[1] and J. NEYTS[1]
[1]Rega Institute for Medical Research and [2]Laboratory of Hepatology, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

We have studied the use of digoxigenin-labelled probes for the detection of HBV-DNA in antiviral drug evaluation. Digoxigenin labelled probes were generated either *via* incorporation of dig-dUTP in (i) a PCR-reaction or (ii) a random priming reaction. The PCR-labelled probes were generated by using the primer set 5'-CTGTGGAGTTACTCTCGTTTTTGC-3' and 5'-CTAACATTGAGATTCCCGAGATTG-3', delineating a 523 bp fragment in the core gene of HBV. After gel purification, this probe was used for the detection of serial dilutions of HBV plasmid DNA immobilized on a Hybond-N membrane. Following a 4 hour to overnight exposure of the hybridized membrane, as low as 1 pg of HBV DNA could be detected. By using a probe labelled *via* random priming with digoxigenin, 5 pg of immobilized HBV plasmid DNA could be detected following overnight exposure on the membrane. In parallel, a ^{32}P-labelled probe was generated by the random priming method. Use of this probe resulted in a detection limit of 10 pg of HBV plasmid DNA following a 2-day exposure. Next, the digoxigenin labelled probes generated *via* PCR or random priming were used to detect HBV DNA in the culture supernatant and in total cellular DNA from HBV-positive hepatoma cells (2.2.15) that had been either treated with selected antiviral agents or that were left untreated. The 50% inhibitory concentrations that were obtained for the antiviral agents lamivudine (3TC), penciclovir (PCV), lobucavir (LBV), adefovir (PMEA) and apropovir (PMPA) were comparable to those published in the literature. Thus, the use of digoxigenin-labelled probes appears to be a simple, convenient, reliable and non-radioactive method for anti-HBV activity assays. In addition, digoxigenin-labelled probes can be stored for > 1 year without loss of activity, and the use of these probes permits shorter exposures of the hybridized membrane than exposure to ^{32}P-labelled probes.

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Effectiveness of combination therapies against Hepatitis B Virus (HBV) replication *in vitro*. Brent E. Korba[1], Chung K. Chu[2], Edgar Hill[3], and Phillip A. Furman[3]. 1-Georgetown Univ. Med. Ctr., Rockville, MD USA; 2-Univ. Georgia, Athens, GA USA; 3-Triangle Phramaceuticals, Inc., Durham, NC USA

Several promising antiviral agents are currently in various stages of clinical trials for the control of chronic HBV infection. However, in the majority of patients, viral replication rapidly returns to pretreatment levels following the withdrawal of therapy. In addition, drug-resistant HBV variants have been observed in significant fraction of patients. Combination therapies with two or more antiviral agents have been shown to enhance antiviral effectiveness against other viral infections (e.g. HIV) as well as in preclinical investigations in the WHV/woodchuck animal model of chronic HBV infection and in pilot clinical studies against HBV. We have investigated the utility of different combinations of nucleoside analogues currently in HBV clinical against HBV replication in 2.2.15 cells. Analysis of drug interactions was evaluated by median-effect plots, Fa-CI plots (with Monte Carlo analysis), and isobolograms. Each combination of antiviral agents was examined at several different molar ratios. Combinations of either 3TC or FTC with penciclovir (PCV) were more effective than the corresponding monotherapies and displayed patterns of antiviral activity that were moderately synergistic at several different molar ratios. Additive to moderately synergistic interactions were generally observed for combinations of 3TC+DAPD, 3TC+L-FMAU, FTC+DAPD, FTC+L-FMAU, DAPD+L-FMAU, and L-FMAU+PCV. In general, a higher degree of cooperativity between the different nucleosides occurred at combinations that theoretically delivered approximately equipotent doses of each nucleoside (based on the results of the monotherapies). Antagonistic interactions were generally found for the combination of DAPD and PCV. In addition, some of the mixtures used for the other combination treatments also exhibited moderate degrees of antagonism, especially when the molar ratios varied significantly from ones that would theoretically deliver equipotent doses. No specific enhancement of cytotoxicity was observed for any of the combinations examined. The patterns of cytotoxicity for the combination treatments were consistent with the profiles observed for the monotherapies. These studies demonstrate that therapies using more than a single antiviral agent can provide an enhanced ability to inhibit HBV replication and provide a basis for the development and design of rational combination treatment regimens to control chronic HBV infection.

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Penciclovir and Mycophenolic Acid Act Synergistically as Inhibitors of *In Vitro* Hepadnaviral Replication. G. Civitico, D. Colledge, S. Locarnini and T. Shaw. Victorian Infectious Diseases Reference Laboratory North Melbourne Victoria, 3051, AUSTRALIA.

The deoxyguanosine analog penciclovir (PCV), which was originally developed as an inhibitor of herpesviruses, is also a potent inhibitor of hepatitis B virus (HBV) replication. Very low concentrations of PCV triphosphate (PCV-TP) are generated intracellularly in hepatocytes by the action of cellular enzymes, since HBV genome does not encode nucleoside kinases. PCV-TP is a poor substrate for cellular DNA polymerases but competes effectively with dGTP as substrate for HBV DNA polymerase/reverse transcriptase, acting as a non-obligate chain terminator; it also inhibits priming of viral minus strand DNA synthesis. Inefficient intracellular phosphorylation is believed to be one of the main factors which limit the anti-HBV of PCV. Mycophenolate Mofetil, an oral prodrug for mycophenolic acid (MPA), which is currently in clinical use as an immunosuppressant, has recently been shown to inhibit HBV replication in primary hepatocytes *in vitro* and to potentiate the antiherpesviral activity of some guanine nucleoside analogs, including penciclovir. MPA acts by inhibiting inosine monophosphate (IMP) dehydrogenase, which results in depletion of intracellular guanine nucleotides and accumulation of IMP. Inhibition of IMP dehydrogenase could theoretically potentiate the antiviral effects of PCV-TP by (1) reducing competition from dGTP and possibly also by (2) increasing the phosphotransferase activity of IMP-GMP-5'-nucleotidase. The latter can use IMP as a phosphate donor and may catalyse the initial phosphorylation of PCV. In primary duckling hepatocyte (PDH) cultures derived from ducklings congenitally infected with the duck hepatitis B virus (DHBV), viral replication was inhibited by approximately 20% by 2.5 μM MPA and by 50% or 90% respectively by 0.1 or 0.5 μM PCV alone. These concentrations were reduced approximately 10- or 3- fold respectively in the presnce of 2.5 μM MPA. Three-dimensional dose-response surface analyses confirmed that MPA and PCV acted at least additively over a wide range of clinically relevent concentrations.

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Myristic acid analogs as potential antiviral compounds.

Panet A., Hodzaev M., Honigman A., and Katzhendler Y.

Faculty of Medicine, The Hebrew University, Jerusalem, Israel.

Fatty acid acylation of specific cellular and viral proteins has been shown to affect the cellular localization and functions of the modified proteins. Myristic acid acylation at the amino termini of both structural and non-structural proteins of several virus families, such as retroviruses, picorna viruses, herpes viruses and hepdna viruses is critical for proper assembly, release and infectivity of the virions. Inhibitors of viral protein myristilation are therefore potential antiviral compounds. However, since myristilation of a subset of cellular proteins is critical for normal cellular functions, effective inhibitors of viral myristilation should demonstrate selectivity. To develop a new class of myristate analogs we have applied computer aided structure modeling to visualize the amino terminus peptide sequence of several myristilated viral proteins. Based on the three dimensional conformation of the myristilated peptide sequences we have designed and subsequently synthesized some thirty analogs of myristate. These analogs were analyzed in several viral systems for their potential inhibitory activities. One group of analogs, characterized by an amide linkage in the fatty acid chain, was inhibitory for the replication of both herpes simplex type-1 and vaccinia viruses in Hela cells. The analog concentrations needed for inhibition of viral replication (IC-50) was in the order of 100μM. Cell toxicity of these analogs is rather low, at 1000μM analog concentration cell proliferation is unaffected. The viral inhibitory concentrations of these lead compounds are clearly too high to be considered as potential drugs for clinical development. Nevertheless, we have applied these inhibitors in studies of virion assembly and maturation. We have also begun to modify these lead compounds in order to enhance their viral inhibitory activity.

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Dendrimers. Novel Antiviral Structures. G. Holan[1], B.R. Matthews[1], E. DeClercq[2], M. Witvrouw[2], J. Neyts[2], B. E. Korba[3], E. R. Kern[4], R. W. Sidwell[5], D.L. Barnard[5], J. H. Huffman[5], Biomolecular Research Institute[1], Melbourne, Victoria, Australia. REGA Institute, Leuven, Belgium[2]. Georgetown University, DMVI, Rockville, MD[3], USA. University of Alabama, Birmingham AL,USA[4]. Utah State University, Logan, UT,USA[5].

Most of drug research, including that on antiviral drugs, concentrates on small molecular weight structures. In contrast, we have investigated large-surface structural frameworks to fill peptide receptors. This resulted in the synthesis of several sets of dendrimers. These are defined as single molecular weight (monodisperse) polymeric entities chemically reacted to grow from a multifunctional core in a controlled manner. The dendrimer layers are synthesised with remaining end functional groups, which are used as reactants for each subsequent "generation" layer leaving again outer functional groups. Each generation has a further multiplicity of branching, until for some structures (e.g. polylysine dendrimer) the steric crowding on the surface of the sphere at their "generation limit" can form a non-draining sphere, which however still retains a surface with reactive functional groups. This results in a molecular core-structure impervious to solvents and a decreased possibility of its recognition by biological systems as an antigen. The functional groups on the surface of the dendrimer's outer layer can be reacted with capping groups recognised by a biological receptor. In this way the size, nature of substitution, volume and nature of the active group can be controlled. Modifying, or biological targeting groups can be reacted on the surface of the active dendrimers to control biological affinity. The surface of several core dendrimers e.g. polyamidoamine (PAMAM), polylysine and novel gallate cores were functionalised with selected surface groups. Unlike previous uses for these structures as polymeric carriers for drugs, antigens etc., the dendrimers could be used as drugs in their own right, due to the properties of the surface coat of the molecular spheres. Many of the anionic aryl surface groups form dendrimer structures with high antiviral activities. Testing in several virology laboratories has demonstrated high, sometime structurally selective activities, against HIV, HBV, CMV, HSV-1 and HSV-2, RSV, Flu-A and Flu-B viruses. The activity, of several classes of dendrimers, cell and tissue penetration and other biological investigations will be presented.

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Effect of Interferon-α B/D Treatment on Viral Parameters in Transgenic Mice Replicating High Levels of Hepatitis B Virus.

R. W. Sidwell[a], K. W. Bailey[a], P. A. Bemis[a], B. A. Korba[b] and J. D. Morrey[a]. [a]Institute for Antiviral Research, Utah State University, Logan, UT, USA. [b]Division of Molecular Virology and Immunology, Georgetown University, Rockville, MD, USA.

The transgenic mice which replicate hepatitis B virus (HBV) in their liver and serum as described by Guidotti et al. (J. Virol. 69:6158, 1995) were used to evaluate the anti-hepatitis effects of interferon-α B/D (IFN-α). Dosages of INF-α of 5 x 10^5 or 5 x 10^6 IU/kg or saline were administered intraperitoneally to transgenic male and female mice (having positive serum HBsAg and HBeAg levels) every other day through a 20-day infection period. Serum was collected on days 0, 7, 14, 21, 28, 35, and 42 and livers were taken on days 0, 21 and 45; the levels of HBV DNA in the serum were evaluated by quantitative PCR; liver HBV DNA was analyzed by Southern blot hybridization. Both doses of IFN-α were highly efficacious in reducing HBV titers in the sera and livers of male mice during time of therapy. Mean day 21 serum titer reductions of 3.7 $\log_{10}$ (below limits of detection) at the high IFN-α dose and 3.0 $\log_{10}$ at the low dose were seen ($P<0.001$). Female mice responded less significantly to the same treatment, with a mean serum titer reduction of 1.6 $\log_{10}$ ($P>0.05$) observed. By day 28, 8 days following termination of therapy, a marked rebound in serum virus titer occurred that was sustained through day 42. Similarly, liver HBV DNA titers were at near-pretreatment values on day 45. The viral titers in placebo-treated animals were not significantly affected at any time in the experiment. These data correlated well with the results of clinical trials using chronically infected HBV patients treated with IFN-α and further validate the utility of this transgenic mouse model for studying potential HBV inhibitors. (Supported by contract NO1-AI-65291 from the Virology Branch, NIAID, NIH).

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PLATINUM(II) COMPLEXES WITH 1-β-RIBOFURANOSYL-1,2,4-TRIAZOLE-3-CARBOXYAMIDE

I. LAKOMSKA[a], E. SZLYK[a], J. KOBE[b]
[a]Nicolaus Copernicus University, Department of Chemistry, Gagarina 7, 87 100 Torun, Poland
[b] National Institute of Chemistry, Hajdrihova 17, 1115 Ljubljana, Slovenia

It is well know that guanine is the reactive site of DNA and interacts selectively with platinum(II) antitumor drugs. The structure of ribavirin (rib) is strikingly similar to that of guanosine, with the carbonyl oxygen and the amide nitrogen occupying stereochemically similar positions to the carbonyl oxygen and the amide ring in guanosine.

The reaction between *cis*-$[Pt(dmso)_2Cl_2]$ and rib in molar ratio M:L=1:1 have been investigate by ^1H, ^{13}C NMR. Direct evidence for the formation of the ionic dinuclear intermediate has been formed and mechanism discussed. Next the *cis* and *trans* complexes of general formula $[Pt(dmso)(rib)Cl_2]$ have been synthesized and characterised by: ^1H, ^{13}C, ^{195}Pt NMR, IR and MS spectroscopy. Spectral analysis results are in favour of Pt(II) in square planar coordination with N(4) bonded ribavirin, S-bonded dimethyl sulfoxide and two chloride atoms in terminal positions. The ^1H resonances of ribavirin revealed the most indicative changes imposed by *cis* and *trans* coordination to Pt(II) out of which the H(5) signals are shifted downfield 0.554 and 0.449 ppm respectively, in relation to free ligand spectrum.

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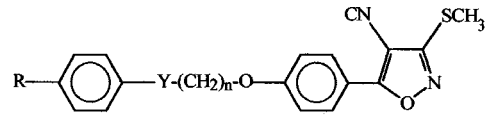
3,4,5 -Trisubstituted Isoxazoles With Antipicornavirus Activity

R.M.Campagna, A.Garozzo°, A.Castro°, G.Tempera°, F.Guerrera* and E.Geremia°°- Departments of Pharmaceutical Sciences*, Microbiological Sciences° and Institute of General Biology°° - University of Catania, Via Androne, 81, 95124, Catania - ITALY*

During the last few years many efforts have been directed toward the identification of agents useful in the prophylaxis and therapy of picornavirus infections. Recently our contribution to the development of new antipicornavirus agents has led to the discovery of 5-aryl-3-methylthio-4-isoxazolecarbonitrile derivatives (Patent 1998, n. MI98A 001072) whose *in vitro* antipicornavirus activity is dependent on the nature of the substituents on the para position of the phenyl ring.

In the present study we investigate the effect of the introduction of bulky substituents on the para position of the phenyl ring on antipicornavirus activity.



Y = CH_2	n = 1, 2	R = H
Y = O	n = 2, 3, 4	R = H
Y = O	n = 2	R = NO_2, COOEt, CH_3, Br, C_6H_5, OC_3H_7

All the compounds were tested against some enteroviruses (polio 1, ECHO 9, coxsackie B_1 and B_3), one cardiovirus serotype (EMC) and 18 HRV serotypes.

The new series of isoxazole derivatives demonstrated an interesting antipicornavirus activity. Our results suggest that the antipicornavirus activity of new derivatives depends on the alkyl chain length: in fact, the obtained compounds exhibited a broad spectrum of activity against a large number of entero and rhinoviruses tested.

Preliminary studies on the mechanism of action suggest that the most active compounds affect some early processes of the virus replicative cycle.

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Isoxazolecarbonitriles: A Novel Class Of Agents Active Against Measles Virus

A.Garozzo°, R.M.Campagna, A.Castro°, G.Tempera°, F.Guerrera* and E.Geremia°°.*

Departments of Microbiological Sciences°, Pharmaceutical Sciences and Institute of General Biology°°- University of Catania, Via Androne, 81, 95124, Catania - ITALY*

In previous studies it was found that 5-aryl-3-methylthio-4-isoxazolecarbonitrile derivatives possessed a promising antipicornavirus activity.

In view of the novelty of this structural class as an anti-RNA virus agent, we examined the effect of the introduction of various substituents on the para position of the phenyl ring on the anti-measles activity.

The first compound, the 3-methylthio-5-phenyl-4-isoxazolecarbonitrile (coded ON-1), was ineffective against measles virus .

The introduction of a -Obut group in the phenyl ring gave an active compound, on the contrary the presence of -OH, -OBn, -OTs groups did not improve the antiviral activity.

Starting from the benzyloxy derivative, the next approach was to elongate the alkyl chain between the two phenyl rings with the aim of preparing compounds with improved anti-measles efficacy.

The new series of isoxazole derivatives demonstrated an interesting anti-measles activity, with high selectivity indexes. The most active member of the series, the 3-methylthio-5-[4-(4-phenoxy-1-butoxy)-phenyl]-4-isoxazolecarbonitrile (coded ON-10), exhibited an IC_{50} of 0.01 μM whereas the 50% cytotoxic concentration was 10 μM.

Preliminary results on the mechanism of action of ON-10 demonstrated an inhibition on some early events of the measles virus replication.

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Ribavirin Antagonises the Effect of Picornavirus Replication Inhibitors.

L. Nikolaeva and A. S. Galabov, Institute of Microbiology, Bulgarian Academy of Sciences, 26, G. Bonchev Str., 1113 Sofia, Bulgaria

The combined effects of ribavirin and a number of picornavirus replication inhibitors with a known mechanism of action on poliovirus type 1 (Mahoney) (PV1) replication in FL cells have been tested. Beforehand, the individual 50% inhibitory concentration (IC_{50}) in the plaque-inhibition test has been determined for each compound, i.e. ribavirin -3 μM, enviroxime - 0.2 μM, disoxaril -0.3 μM, arildone -2.7 μM, S-7 - 100 μM, guanidine.HCl - 200 μM, PTU-23 -200 μM and HBB - 300 μM. Combining ribavirin with each of the above mentioned inhibitors results in a marked antagonism. In order to reveal the reason for that antagonism the effect of the combination ribavirin +MCU [1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone], a specific togavirus inhibitor, has been tested on the replication of Semliki Forest Virus (SFV), whose genome is also a plus-RNA one but it is "capped" in contrast to that of picornaviruses. The combination has turned out to be a synergistic one, which could be explained by the attack on two different targets in the replicative cycle of SFV. Comparison of the experimental results on both virus models leads to the suspicion that a "cap"-independent, most probably cell-mediated mode of action of ribavirin could be responsible for the observed antagonistic combined effects against the replication of VP1 (Mahoney).

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Antiviral efficacy of VX-497, a novel IMPDH inhibitor, or ribavirin in combination with Interferon α in Encephalomyocarditis virus (EMCV) infected L929 cells.

W. Markland, T. McQuaid and A.D. Kwong. Vertex Pharmaceuticals Inc. 130 Waverly St. Cambridge, MA 02139 USA

VX-497 is a novel inosine monophosphate dehydrogenase (IMPDH) inhibitor which was developed by structure-based drug design utilizing the crystal structure of IMPDH. Inhibitors of IMPDH block the purine nucleotide biosynthesis *de novo* pathway and have been shown to have antiviral and immunosuppressive activity via reduction of the intracellular guanine nucleotide pools required for RNA and DNA synthesis. Ribavirin is a broad-spectrum antiviral agent with activity against a number of DNA- and RNA-containing viruses and one of its possible mechanisms of action is through IMPDH inhibition. Ribavirin in combination with IFNα has recently been approved for the treatment of Hepatitis C virus infection. We initiated experiments to determine if VX-497 has antiviral activity, and whether this activity is increased when combined with IFNα. Using Encephalomyocarditis virus (EMCV) and mouse L929 cells in a 96 well format, we set up a simple and rapid viral infection assay in which the antiviral activity of test compounds was indicated by a reduction in the cytopathic effect. This was measured using the conversion of a tetrazolium compound (MTS) in test cultures as compared with cell and viral controls. Initial validation of the system using IFNα alone gave an IC_{50} value of 0.95 unit, a value comparable to the manufacturer's specification. The antiviral activity of ribavirin was confirmed, giving an IC_{50} in the range of 18 to 50μM in this system, and was shown to be additive when co-administered with IFNα. VX-497 was demonstrated to have a more potent antiviral activity, with IC_{50}'s in the range of 0.5 to 3μM and was similarly shown to present an additive antiviral effect in the presence of IFNα. Further studies of VX-497 antiviral activity using a variety of cell lines and viruses will be presented.

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Mode of Action of Ribavirin Against Cowpox and Monkeypox Viruses.

Donald F. Smee and John W. Huggins. Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

Ribavirin (Rbv) is inhibitory to poxviruses, and may be useful in treating severe infections in humans. The drug is metabolized intracellularly to mono-, di-, and triphosphate forms (RMP, RDP, and RTP). Each metabolite possesses inhibitory activity against certain viral or cellular enzymes. It has been hypothesized that poxviruses are inhibited by RTP's effects on virus-specific mRNA capping functions. Alternatively, inhibition of cellular inosine monophosphate dehydrogenase (IMPdH) by RMP may be more important, since an unrelated inhibitor of IMPdH, mycophenolic acid (MPA), is a potent inhibitor of poxviruses. IMPdH inhibition leads to suppression of guanosine triphosphate (GTP) levels, causing inhibition of RNA synthesis and protein synthesis. In plaque reduction assays, Rbv was 15- to 30-fold more active in mouse 3T3 cells than in monkey Vero cells. Monkeypox virus was 3- to 7-fold more sensitive to Rbv than cowpox virus. Comparisons were made of the degrees of virus yield or plaque reduction to the extent of GTP suppression by Rbv and MPA in the different cell lines and against the two viruses. A 100-fold higher concentration of Rbv than MPA was required to suppress GTP pools by 50% in Vero cells, resulting in 90% inhibition of virus yield with either drug. A 20-fold higher concentration of Rbv was needed to suppress GTP pools in Vero cells compared to 3T3 cells. At multiple levels of GTP suppression the extent of inhibition of virus replication was the same in the two cell lines. To explain why Rbv was more active in 3T3 cells than in Vero cells, the intracellular metabolism of radioactive drug was studied. At equal extracellular drug concentrations, approximately 13-fold more RMP was formed in 3T3 cells than in Vero cells, corresponding to the plaque reduction potency differences of Rbv for poxviruses in the cell lines. Based on these correlations, the results are consistent with the hypothesis that the inhibition of cellular IMPdH contributes significantly to the anti-poxvirus activity of ribavirin.

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Nucleoside phosphonates cidofovir and adefovir are substrates for human renal organic anion transporter.

Tomas Cihlar[1], John B. Pritchard[2], Michael D. Fuller[1], Deborah C. Lin[1], and Douglas H. Sweet[2]. [1]Gilead Sciences, Foster City, CA 94404, USA; [2]National Institute of Environmental Health Science, Research Triangle Park, NC 27709, USA.

Nucleoside phosphonates cidofovir and adefovir are potent antiviral agents with unique drug resistance profiles. The most important clinical and preclinical toxicity of these drugs is nephrotoxicity detected by changes in laboratory markers of renal functions. Preclinical studies showed accumulation of both drugs in the kidney. To ameliorate the nephrotoxicity of cidofovir, it is coadministered with probenecid, which decreases its active renal excretion thereby reduces its accumulation in proximal convoluted tubules of the nephron. Since the active renal accumulation of cidofovir and adefovir likely plays a role in the etiology of these adverse effects, we attempted to identify a renal transport system capable of interacting with these antiviral agents. Recently, the expression cloning of a probenecid-sensitive basolateral rat renal organic anion transporter (rROAT1) has been reported (Sweet *et al.*, J. Biol. Chem. 272, 1997). After the screening a human kidney cDNA library with a rROAT1-derived probe, we isolated a cDNA encoding the human renal organic anion transporter (hOAT1), a 550 amino acid polypeptide with 12 putative transmembrane domains and 86% sequence homology with rROAT1. The 2.6 kb hOAT1 mRNA is abundant in kidney, but has not been detected in other human tissues. Expression of hOAT1 in *Xenopus laevis* oocytes injected with hOAT1 cRNA resulted in probenecid-sensitive uptake of p-aminohippuric acid (the prototype substrate for the classical kidney organic anion transporter systems) at levels similar to that of rROAT1. Notably, hOAT1 also mediated probenecid-sensitive uptake of cidofovir and adefovir. A comparable capacity to transport p-aminohippuric acid, cidofovir and adefovir indicates that hOAT1 may significantly contribute to the active accumulation of nucleoside phosphonates in kidney tissue. Further characterization of hOAT1 may help to better understand the molecular aspects of nucleoside phosphonate nephrotoxicity and suggest an approach for its effective management.

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Monitoring the Effect of Nucleosides and Their Analogs on Nucleotide Pools by Capillary Electrophoresis.

D.D.Y. Chen, C. Luscombe, G. Bebault, and S.L. Sacks The University of British Columbia and Viridae Clinical Sciences, Inc., Vancouver, Canada

Nucleoside antivirals (NA) continue to play a key role in treating viral infections. Therapy may influence both intracellular anabolism of NA phosphates (NAP) as well as changes in natural competitors in endogenous nucleotide pools (ENP). Furthermore, very little is known about alterations in ENP under the influence of NA combinations. Capillary electrophoresis (CE) is a promising technique which offers enough separation power for the detailed studies of the amount of each NA, NAP, and ENP in cell extracts. In order to further understanding of changes of NA, NAP, and ENP with and without the influence of NA's, we developed a CE assay which does not require radioactivity ENP studies. For initial studies, cytosol extracts from approximately 25 million mouse lymphoma cells were prepared. Cell pellets were extracted with 50% ethanol, 10% PBS (1 mL) containing an internal standard, deoxyinosinemonophosphate (1 mg/100 mL). Extracts were evaporated to dryness and redissolved in deionized water. CE separated approximately 30 components. Using this modified CE analysis, all ribo and deoxyribo ENP's were detected. The most abundant ENP was AMP with an extract concentration of about 1mM. Surprisingly, ribonucleotide pools were profoundly affected by treatment of uninfected cells with exogenous NA's and deoxyguanosine, reducing intracellular concentrations of both adenosine and guanosine ribonucleotides to ≤ 10% of untreated cells, while dGTP levels increased. CE analysis has the advantage of concentrating specific ENP's and NAP's during the assay, thereby enhancing distinct separation. Conditions of treatment and infection, as well as host cell can be modified as required. Further studies are required to exploit the potential for this system in enhancing our understanding of the mechanisms of NA's singly, and in combination.

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Evaluation of the Cross-Resistance Profiles of Lamivudine- and Famciclovir-Resistant Hepatitis B Viruses and Sensitivity to Adefovir *In Vitro*. X Xiong, H Yang, C Westland, R Zou, and C Gibbs. Gilead Sciences, Foster City, CA, USA.

Two nucleoside analogs, lamivudine and famciclovir have displayed efficacy against HBV in clinical studies. However, the emergence of resistance has limited the efficacy of these agents during extended treatment. In HBV isolated from lamivudine-resistant patients, mutations (M552I, M552V, L528M+M552I & L528M+M552V) have been found in conserved subdomains B and C of HBV polymerase and have been demonstrated to cause resistance to lamivudine in enzymatic and cell culture models *in vitro* and in animal models. Additional mutations including V521L, P525L, L528M, T532S, and V555I, have been reported in HBV isolated from patients that displayed HBV break-through during famciclovir treatment. However, these mutations have not yet been directly correlated with a famciclovir resistant phenotype in experimental systems. A nucleotide analog, adefovir dipivoxil is in clinical trials for the treatment of HBV and HIV infections. Adefovir demonstrated a potent dose-dependent anti-HBV effect in chronic hepatitis B patients treated for 12 weeks (median viral load was reduced by 4 logs or greater in the 30 and 60 mg daily dose groups). No resistance to adefovir was observed during 12 week dosing. In order to evaluate the cross-resistance profiles of these emerging new therapies we compared the inhibition constants (K_i) of the active metabolites of adefovir, lamivudine, and famciclovir for recombinant wild-type and mutant human HBV polymerases in an *in vitro* polymerase assay. In the enzymatic assay, the M552I, M552V & L528M+M552V mutations showed strong resistance to lamivudine with K_i values increasing by 8 to 25-fold. The V555I mutation displayed the most resistance (6.2-fold) and several other mutations caused moderate resistance to the active form of famciclovir. Some mutations caused moderate cross-resistance between lamivudine and famciclovir, however, all the mutations remained sensitive to adefovir with K_i values increased by less than 2.3-fold. Ongoing studies include; the analysis of additional mutations associated with lamivudine and HBIG-resistance, the evaluation of other novel anti-HBV compounds and analysis of resistance and viral replication competence in a cell culture model of HBV replication. In conclusion, adefovir may be beneficial for the treatment of chronic hepatitis B patients, including those patients who have failed lamivudine or famciclovir therapy.

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Antiviral activity of Clevudine (L-FMAU) against WHV replication and gene expression in chronically-infected woodchucks. Brent Korba[1], Simon Peek[2], Ilia Toshkov[2], Paul Cote[1], Chung K. Chu[3], James Jacobs[2],John Gerin[1], and Bud Tennant[3], 1-Georgetown Univ. Med. Ctr., Rockville, MD USA; 3-College of Vet. Med., Cornell Univ., Ithaca, NY USA; 3-Univ. Georgia, Athens, GA USA;

The woodchuck hepatitis B virus (WHV) and its natural host, the Eastern woodchuck (*M. monax*) are a valuable model for HBV-induced disease, including HCC, and a predictive model for HBV response to antiviral therapy. We have examined the antiviral effectiveness of L-FMAU (Clevudine, 1-(2'-deoxy-2'-fluoro-*B*-L-arabinofuranosyl)-5-methyluracil) against chronic WHV infection. L-FMAU induced an abrupt, progressive, dose-related suppression of viremia and intrahepatic WHV replication. Once daily, oral, doses as low as 0.3 mg/kg were effective, and at 10 mg/kg viremia was depressed over 200-fold in 48 hours, and up to 1 billion-fold after 4 weeks. Following drug withdrawal, a dose dependent return in viremia was observed. Viremia in 2 animals receiving 10 mg/kg remained depressed up to 25 weeks post-treatment. In another study, doses of 10mg/kg for 12 weeks induced reductions of WHV replication, serum WHsAg, and hepatic WHcAg that were sustained in 3 of 4 animals for 68 weeks post-treatment. WHV markers in the fourth animal (WC5299) remained suppressed for approximately 20 weeks post-treatment and gradually returned to pre-treatment levels. The 12 week treatment reduced serologic and histologic markers of WHV-induced liver disease, and did not induce any obvious indications of toxicity. Analysis of WHV cccDNA in the terminal liver samples showed a marked reduction in 3 of 4 animals (cccDNA in WC5299 was in the range observed for untreated WHV carriers) indicating that the likely mechanism of action for L-FMAU is an inhibition of virus replication below the level necessary to maintain cccDNA, resulting in a progressive loss of virus-infected cells from the liver. The capacity to induce such a chain of events indicates that lifetime therapy with L-FMAU, or any other equally effective antiviral regimen, may not be necessary to control HBV infection. We are currently conducting longer term therapy trials to further assess the antiviral efficacy of L-FMAU and to examine the possibility of drug-resistance. We are also attempting to identify specific host factors that may be correlated with the control of WHV replication during and following therapy. L-FMAU is a highly effective agent against WHV replication and, as such, has potential use for the control of chronic HBV infection.

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The Trimera mouse: an HBV and HCV infection model for evaluation of antiviral therapeutic agents.

S. Dagan[1], O. Nussbaum[1], R. Eren[1], O. Ben-Moshe[1], Y. Arazi[1], S. Berre[1], I. Lubin[1], D. Shouval[2], E. Galun[2], Y. Reisner[3] and E. Ilan[1]. XTL Biopharmaceuticals, Kiryat Weizmann, Rehovot,[1] Hadassah University Hospital, Jerusalem[2] and the Weizmann Institute of Science,[3] ISRAEL.

One of the major difficulties in developing therapies against hepatitis C and hepatitis B is the lack of a reproducible small animal model system for preclinical evaluation of therapeutic candidates. We have generated the Trimera mouse system in which normal mice, lethally irradiated and radioprotected with SCID mouse bone marrow cells, were transplanted under the kidney capsule with HCV or HBV *ex vivo* infected human liver fragments. Viremia was assessed by PCR or RT-PCR followed by dot blot hybridization. Engraftment of viable liver fragments was observed in 85% of the transplanted animals one month after transplantation, as evaluated by H-E staining and by the presence of HSA mRNA in the grafted tissues. HBV-DNA or HCV-RNA can be detected 8 days after liver transplantation and the levels peak between days 18 and 25. Infection rate reaches 85% at the peak of viremia. The presence of HBV-cccDNA or (-)strand HCV RNA indicate viral replication in the Trimera model. The HBV-Trimera model was used to test the therapeutic effects of human polyclonal anti-HBs antibodies (Hepatect^{R}) and the polymerase inhibitors 3TC and β-L-5FddC. Treatment of HBV-Trimera mice with these drugs reduced both the percentage of animals infected and the viral load in their sera. The HCV-Trimera model was used to test effect of anti-HCV antibodies as well as other potential anti-HCV agents. The Trimera mouse model may offer an effective tool for simulating human HCV or HBV infection and for evaluating new therapeutic agents.

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Generation of high affinity, fully human monoclonal antibodies specific to hepatitis B virus in a human/mouse radiation chimera: the Trimera system.

R. Eren*, I. Lubin*, D. Terkieltaub*, O. Ben-Moshe*, A. Zauberman*, R. Uhlmann*, T. Tzahor*, S. Moss*, E. Ilan*, E. Galun+, Y. Reisner# and S. Dagan*. *XTL Biopharmaceuticals Ltd., Kiryat Weizmann, Rehovot, +Liver Unit, Hadassah University Hospital, Jerusalem, #Dept. of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.

We have generated the Trimera mouse system in which normal strains of mice, lethally irradiated by total body irradiation and radioprotected with SCID mouse bone marrow cells, are rendered permissive for engraftment of human cells and tissues. To obtain human mAbs to HBV, Trimera mice were transplanted with lymphocytes from donors positive for anti- hepatitis B surface antigen (HBsAg) antibodies and subsequently immunized with hepatitis B vaccine. Two weeks after immunization, spleens of the responding Trimera mice were harvested and cells fused to human-mouse heteromyeloma cells to generate hybridoma clones. Several stable clones secreting IgG specific for HBsAg were isolated and their antibodies were characterized. These mAbs are IgG1 and have high affinity constants for HBsAg in the range of 10^{-10} M. Two of these mAbs, HBV-AB17 and HBV-AB19 were further developed and characterized. Specificity to HBsAg was tested by a competitive inhibition assay and by immunohistostaining of human liver infected with HBV. The two antibodies bind to different epitopes on the *a* determinant of the HBsAg and to all viral subtypes with distinct patterns, indicating diversity and broad reactivity. The biological function and neutralizing activity of these antibodies are being tested.

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A Transgenic Mouse Lineage Useful for Testing Antivirals Targeting Hepatitis B Virus.

P.L. MARION*, F.H. SALAZAR, K. LIITTSCHWAGER, B.B. BORDIER, AND A.D. COOPER.
Stanford University, Stanford CA; Hepadnavirus Testing, Inc., Mountain View CA; Palo Alto Medical Foundation, Palo Alto CA.

Guidotti et al (J. Virol. 69:6158, 1995) have made hepatitis B virus (HBV)-producing transgenic mice whose levels of viremia are similar to those found in chronically-infected patients. Additional small animal models with which therapeutic approaches against human HBV can be evaluated are needed to provide greater availability of testing and confirmation of findings in the original HBV-producing mice. Using a similar HBV DNA construct, we have made a lineage of transgenic mice which has serum HBV DNA levels of 10^6 to 10^8 viral genome equivalents (vge)/ml in 70-90% of offspring tested at 30 days of age, with the remainder producing less virus. During the next 1-3 months, viral DNA and HBsAg levels of these mice decline slowly (approximately two-fold). Serum levels of HBsAg and HBeAg are proportional to the viral DNA levels, and there does not appear to be a difference in HBeAg levels between male and female mice. An endogenous DNA polymerase activity is present in particles pelleted from serum, and the product of this reaction migrates on agarose gels like HBV DNA from human serum, indicating that whole virus is present in the mouse serum. BMS-200475, kindly provided by R. J. Colonno of Bristol-Myers Squibb, is a cyclopentyl 2'-deoxy-guanosine nucleoside which is in clinical development for treatment of chronic HBV infections. It inhibits HBV replication in 2.2.15 cells and is active against woodchuck hepatitis virus in woodchucks and duck hepatitis B virus (DHBV) in ducks. To evaluate the effect of the drug in the HBV transgene model, 6 one month-old STC mice were given 1 mg/kg/day BMS-200475 by intraperitoneal injection for 20 days. Another group of mice received vehicle alone. The average reduction of viral DNA levels in the BMS-200475-treated mice at the end of treatment was 40 fold, while that of vehicle-treated was 4 fold. These results support use of this model system to test the efficacy of inhibitors of viral DNA replication.

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Prolonged Lamivudine Therapy for Chronic Hepatitis B: Safety, Efficacy on HBV, Variant Emergence, and Seroconversion.

Bourne, E.,[1] Gauthier, J.,[1] Crowther, L.,[1] Dienstag, J.,[2] Brown, N.,[1] and Condreay, L[1]. Glaxo Wellcome Inc., Research Triangle Park, NC 27709, [2] Massachusetts General Hospital, Boston, MA 02114

Lamivudine [(-)2'-deoxy-3'-thiacytidine, 3TC], a nucleoside analogue, is a potent inhibitor of hepatitis B virus (HBV) reverse transcriptase. In this study, twenty-four patients participated for up to 18 months in a Phase II, open-label, trial of lamivudine for chronic HBV infection. One patient did not continue past baseline. Clinical parameters examined included safety/tolerance, HBV DNA suppression as measured by a solution hybridization assay, and HBeAg loss/seroconversion. Parameters examined in a virology sub-study of 23 patients with multiple sample points included HBV viremia, emergence of lamivudine-resistant variants at the $YM_{552}DD$ locus of the HBV polymerase, and HBeAg seroconversion. Serum ALT level improvements accompanied HBV DNA suppression. Serum HBV DNA became undetectable in all patients using the solution hybridization assay. Cumulative loss of HBeAg occurred in 10/24 (42%), was confirmed in 9/24 (38%), and was accompanied by HBeAb in 5/9 patients with sustained HBeAg loss. Serologic status was maintained in 6/7 patient in which therapy was discontinued after HBeAg loss/seroconversion. Using a more sensitive PCR-based assay, viral load remained > 10^4 genomes/mL in 11 patients and decreased to < 10^4 genomes/mL in 12 patients. Ten patients acquired $YM_{552}DD$-variant HBV during treatment. An additional HBV variant, L528M, was often associated with the M552V variant. Six patients with HBV DNA levels < 10^4 genomes/mL, including 3 patients with $YM_{552}DD$ variants, seroconverted during the treatment period or in the follow-up period after treatment discontinuation. No patients with HBV DNA levels > 10^4 genomes/mL seroconverted during the treatment phase or the post-treatment follow-up period. These results suggest that patients with dramatic reductions in viral load (to levels < 10^4 genomes/mL) due to lamivudine therapy might be more likely to seroconvert than patients with modest reductions and that development of lamivudine-resistant HBV variants does not prevent seroconversion in patients with dramatic reductions in viral load.

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Biological Characterization and Peptide-Based Inhibition of the Hepatitis C Virus NS3 Serine Protease

Daniel Lamarre, Boehringer Ingelheim (Canada) Ltd., Bio-Méga Research Division, Laval, Québec, Canada.

Hepatitis C Virus (HCV) has become one of the most important etiological agents worldwide in the development of chronic liver diseases. Studies of the molecular biology of HCV have yielded critical insight into the virus-specific components that represent attractive targets for drug discovery. Our initial efforts have focused on inhibition of the HCV serine protease, an enzyme that plays a central role in the life cycle of the virus. The NS3 protease domain (N-terminal 180 amino acids) and heterodimeric NS3-NS4A complex were purified to homogeneity and used for assay development and lead discovery. Kinetic studies using an NS5A/5B-derived fluorogenic peptide substrate will be presented. Inhibition studies showing that the N-terminal cleavage product DDIVPC-OH of a peptide substrate was a competitive inhibitor of the reaction provided a rational approach for the design of substrate-based inhibitors. SAR studies based upon this peptide lead have resulted in inhibitors of the NS3-NS4A protease activity with submicromolar potency. Studies describing the contribution of the C-terminal carboxylic acid in binding and specificity of these inhibitors will be discussed. The solution structure of a bound inhibitor determined by NMR revealed a well-defined extended conformation upon enzyme complexation. These peptide-based molecules are promising leads for the development of novel therapeutics in HCV disease.

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X-ray Crystal Structure of the Antiviral Drug Ribavirin Monophosphate Bound to IMP Dehydrogenase

M.D. Sintchak, M.C. Badia, O. Futer, and E. Nimmesgern
Vertex Pharmaceuticals, 130 Waverly Street, Cambridge, MA, USA

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated 170 million people worldwide who are infected. In the United States alone, over 4 million people are infected with HCV, and approximately 30,000 new cases and 10,000 deaths are reported each year. The course of this potentially lethal disease varies greatly among those infected. While some patients experience no symptoms for decades, a sizeable fraction eventually develop cirrhosis, end-stage liver disease, and even liver cancer. There is no vaccine against HCV, and current front-line therapy involves treatment using interferon alpha alone or in combination with ribavirin. Some shortcomings of this combination therapy are that less than 50% of the patients are responsive, and ribavirin causes nonimmune hemolytic anemia. While ribavirin has been known for years to exhibit antiviral activity against several RNA and DNA viruses, its exact mode of action is poorly understood. One possible mechanism is the depletion of intracellular guanosine nucleotide pools through the direct inhibition of inosine-5'-monophosphate dehydrogenase (IMPDH). In order to better understand the molecular details of IMPDH inhibition by ribavirin, we have synthesized ribavirin monophosphate and determined the X-ray crystal structure in complex with a deletion mutant of human IMPDH type II at 1.85 Å resolution. Since ribavirin monophosphate is a competitive inhibitor of IMPDH, it binds to the substrate (IMP) binding portion of the active site. The structure reveals that ribavirin monophosphate is an excellent substrate mimic and superimposes perfectly with IMP. Interestingly, a flap from residues 420 through 437 adopts a new conformation in this structure such that it now occupies the cofactor (NAD) binding portion of the active site where uncompetitive inhibitors such as mycophenolic acid and VX-497 bind. This new structural information should help guide efforts to design new inhibitors of IMPDH which may serve as anti-HCV drugs with improved potency and fewer side effects than existing therapies.

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DAPD: A Novel Inhibitor of Hepatitis B Virus Replication. G. R. Painter, L.L. Kiefer, R.F. Schinazi, B. Korba, C.K. Chu, B. Tennant, E.L. Hill, J. Begley, B. Lampert, S. Locarnini, and P.A. Furman. Triangle Pharmaceuticals, Inc. Durham, NC USA.

(-)-β-D-2, 6-diaminopurine dioxolane (DAPD) is a selective and potent inhibitor of HBV and HIV *in vitro*, and has been demonstrated to be a potent inhibitor of WHV *in vivo*. DAPD is deaminated *in vitro* and *in vivo* by adenosine deaminase to give (-)-β-D-dioxolane guanine (DXG), which is also a potent inhibitor of HBV replication. Using purified calf adenosine deaminase, a K_m for DAPD of 11μM, and a K_{cat} of 0.25 s^{-1} was measured. The EC_{50} values for DAPD and DXG against HBV in the 2.2.15 cell assay are 0.023 ± 0.002 μM and 0.16 ± 0.014 μM, respectively. To determine the source of differential activity between DAPD and DXG, studies are underway to measure intracellular levels of 5'-phosphorylated intermediates. The 5'-triphosphosphates of DAPD and DXG are both inhibitors of the HBV polymerase with IC_{50} values of 0.07 μM. and 0.04 μM, respectively. Because pharmacokinetic studies have shown that DAPD is converted to DXG *in vivo*, an understanding of the factors that effect the relative activity of DAPD and DXG is important to further drug development.

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Response to Therapy with Adefovir Dipivoxil is Durable for 48 Weeks and Correlates with Baseline HIV Reverse Transcriptase Genotype as well as Baseline In Vitro Susceptibility to Adefovir

M.D. Miller, K.E. Anton, N.A. Margot, P.D. Lamy, A.S. Mulato, and J.M. Cherrington, Gilead Sciences, Foster City, CA, USA

GS-96-408 was a phase III clinical trial assessing adefovir dipivoxil (ADV) compared to placebo for the treatment of HIV-1 infection. 142 patients were prospectively selected for a virology substudy. At week 24, the ADV-treated patients in the substudy showed a statistically significant (*) mean 0.53 $\log_{10}$ decrease in plasma HIV RNA versus an 0.01 $\log_{10}$ increase for placebo patients ($p < 0.0001$). These responses were durable in patients who continued ADV therapy for up to 48 weeks. Patients were grouped according to their baseline RT resistance genotype:

3TC Resistance Mutations	AZT Resistance Mutations	Number of Patients in Group (%)	Mean $\log_{10}$ Δ in HIV RNA at Week 24	
			ADV	Placebo
none	none	9 (6%)	-0.65	-0.11
none	low	5 (4%)	-0.65	+0.23
none	high	20 (14%)	-0.05	+0.05
M184V	none	21 (15%)	-0.94*	+0.09
M184V	low	12 (8%)	-0.75*	+0.07
M184V	high	75 (53%)	-0.51*	-0.04

Thus, 86% of patients were in the 5 responsive groups, including the largest group of patients with both high-level AZT and 3TC resistance mutations. Although RT mutations did arise during therapy, these RT mutations were not specific to ADV and were not associated with increases in HIV RNA. Phenotypic analyses were also performed with baseline RT recombinant viruses from 28 patients representing the 6 genetic groups. Recombinant viruses from patients in the 5 responsive groups showed IC_{50} values for adefovir that were wild-type or within 3-fold of wild-type; whereas viruses from the single non-responsive group were >5-fold above wild-type. These data suggest that both genotypic and phenotypic testing can be used to predict response to adefovir dipivoxil therapy in patients on a stable antiretroviral regimen.

Oral Session VII: Herpesvirus Infections II

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Dihydropyridines: A New Class of Potent Non-nucleoside Inhibitors of HSV TK

J A Martin, G M Taylor, S J Baker, M J Holland, J H Merrett, B S Sherborne, G E M Sibley, A Walmsley and P Wong-Kai-In
Roche Discovery Welwyn, Broadwater Road, Welwyn Garden City, Hertfordshire AL7 3AY, UK

Previously, we have reported on the rational design and synthesis of nucleoside analogues as potent and selective inhibitors of herpes simplex virus thymidine kinase (HSV TK) which demonstrated good antiviral activity in cell culture and in animal models of infection. In an approach to discover novel inhibitors of HSV TK we adapted a biochemical assay to high throughput screening (HTS) format and identified a dihydropyridine 'hit' with an IC_{50} value of 64nM against HSV-2 TK. Further mining of the compound libraries afforded related analogues with improved activity indicating that potency of the HTS 'hit' could be substantially improved. Another attractive feature of this series was the ease of synthesis from readily available starting materials. A chemical programme was initiated generating new analogues which improved enzyme inhibitory activity. Ro 32-6882, a representative example from the series of optimised analogues, is active at the sub nanomolar level against both HSV-1 and HSV-2 TK. It is selective for the viral enzymes, inhibits the reactivation of latently infected murine ganglia in cell culture and is orally bioavailable. This presentation will describe the lead optimisation process and structural features that are important to impart oral bioavailability in this dihydropyridine series.

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Differences in Pathogenicity of Herpes Simplex Virus (HSV) Strains as Observed by High-Resolution Cranial Magnetic Resonance Imaging (MRI) and Histopathology in a Murine Encephalitis Model.

R. Tal-Singer, H. Thomas, G. Wells, A. Gresham, H. Solleveld, D. Sutton, K. Esser, S. Dillon, S.Sarkar, R. Kapadia. SmithKline Beecham Pharmaceuticals, Collegeville, Pennsylvania, USA.

HSV induced encephalitis in the mouse is an established pre-clinical model for evaluating different treatment modalities. There have been some reports describing the use of MRI as a tool to monitor the development of Herpes simplex induced encephalitis (HSE) in the murine brain. However, the correlation with histopathological images has not been well documented, possibly due to resolution of the MR images and choice of imaging times. We have used high resolution MRI (4.5T) to follow the progression of HSE during the first week of infection in a murine model. Female BALB/c were inoculated intracerebrally with 100 PFU of HSV-1 or HSV-2. Each animal was evaluated daily by T2 weighted MRI and recording of clinical status. In addition, contrast enhanced images were obtained from mice at various time points. Brain samples were collected on days 2 or 4 post-infection and analyzed for histologic lesions and viral antigen expression. Although both HSV strains caused similar clinical disease patterns, we observed significant differences in MR images as well as histopathological findings. HSV-1 MR pathology: ventricular enlargement and hyperintense regions around the hippocampus were discernible within 1-2 days following infection and increased in severity (size) paralleling clinical disease progression. In contrast, changes caused by HSV-2 were significantly milder and restricted to the meningeal regions only in a few mice. The changes observed in the MR images were confirmed by light microscopic examination. HSV-1 associated histologic lesions consisted of necrotizing meningoencephalitis whereas HSV-2 lesions were confined to the meninges. HSV-1 antigen staining was widespread whereas HSV-2 antigen was mostly limited to the meninges. Similar differences were observed in nude mice suggesting that the changes were not due to T-cell mediated response. In conclusion, serial in vivo MR imaging may be useful to evaluate HSE -related changes and the efficacy of anti-infective therapeutic approaches in individual animals.

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The Cellular Location of Transient Virus Production in Neural Tissues on Cessation of Chemotherapy Using Valaciclovir.

A.M. Thackray, N.M. Field & H.J. Field
Centre for Veterinary Science, Cambridge University, Madingley Road, Cambridge, UK. CB3 0ES.

We have reported previously using both HSV-1 (JID, 173, 291,1996) and HSV-2 (Antimicrob. Ag. Chemother, 40, 846, 1996) that, after a continuous period of valaciclovir (VACV) therapy, when treatment is terminated, infectious virus may be detected in neural tissues within 1 to 5 days following drug withdrawal. We designed an experiment to investigate the nature of this transient recurrence of infectious virus. We used a recombinant strain of HSV-1 (SC16) containing the β-galactosidase reporter gene under the IE110 promoter (Efstathiou & Lachmann, pers. com.). Mice were inoculated in the ear pinna. SC16 (IE110) was markedly attenuated and few clinical signs were evident during the acute phase of the infection; none of the mice developed neurological signs, and none died. VACV was supplied in the drinking water (approx. 150 mg/Kg/day) from day 1 to 8 or 1 to 11 p.i. and some were untreated. Mice were killed daily from day 7 to 13 p.i.; and tissue sections prepared from brain and ganglia. *In situ* hybridisation using DIG-labelled riboprobes for ICP0 and major LAT, showed that a large number of trigeminal ganglion neurons, some cervical neurons, and many scattered cells in the brain stem were positive. The intensity of nuclear stain varied but the pattern of staining was distinctive. Similar material was obtained from untreated mice. Controls included known latently infected tissues, uninfected tissues and hybridisation using opposite strand probes. The distribution of the cells showing a positive signal by *in situ* hybridisation was compared with the distribution of positive cells labelled by X-gal, the reporter gene product. This revealed isolated, blue, positive-staining neurons in the ganglia and small aggregates of blue cells in the region of the sensory root of the 5th nerve in the brain stem. The infected cells were unequivocally identified as neurons. A few blue-staining neurons were detected at late times in ganglia from untreated mice suggesting that virus production continues during a phase when infectious virus cannot be detected using conventional culture techniques and this may help explain the transient infectious virus seen on cessation of chemotherapy.

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S-acetylglutathione inhibits viral replication and potentiates antiviral effects of aciclovir against herpes simplex virus type 1 and varicella zoster-virus *in vitro*

J.-U. Vogel[1,2], H. Rabenau[1], A. Korb-Bangang[1], M. Scholz[1], G. Treusch[3], H.W. Doerr[1], J. Cinatl Jr.[1]

[1]Institute of Medical Virology, [2]Center of Pediatrics, Dept. of Hematology and Oncology, Johann Wolfgang Goethe-University, Sandhofstr. 2-4, D-60528 Frankfurt/M.; [3]Syncomp GmbH, Brühlstr. 37, D-60439 Frankfurt/M., Germany

Intracellular glutathione (GSH) plays an important role in the regulation of replication of different viruses including human herpes viruses. A novel GSH derivative S-Acetylglutathione (A-GSH) was developed to provide an agent which (in contrast to GSH itself) may be transported into cells and intracellulary converted to GSH. We tested effects of A-GSH against human herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV) in cultures of human dermal fibroblasts (HDF) and retinal pigment epithelial (RPE) cells. A-GSH at non-toxic concentrations ranging from 5 to 20 mM suppressed virus replication in a dose dependent manner as demonstrated by measurements of virus load. In HDF infected with HSV-1 virus load was reduced at least 100-fold while VZV was inhibited only 2- to 3-fold in RPE cells. A-GSH addition to HSV-1 infected cultures rapidly resulted in increased intracellular content of GSH and total thiol groups which decreased immediately after virus infection. A-GSH showed strong synergistic effects in a combination with aciclovir both against HSV-1 and VZV. The results demonstrate that A-GSH is a potent antiviral agent against HSV-1 and VZV which may be due to its ability to restore intracellular GSH levels. Strong synergistic antiviral effects of A-GSH with aciclovir suggest that A-GSH may be of benefit for the adjunctive treatment of HSV-1 or VZV infection.

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Novel Hydrogels with Microbicidial Activity Prevent Intravaginal and Intracutaneous Infections with HSV-2 in a Mouse Model: impact on the search for vaginal microbicides

J. NEYTS[1], T. KRISTMUNDSDOTTIR[2], E. DE CLERCQ[1] and H. THORMAR[3]
[1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium; [2]Department of Pharmacy, University of Iceland, Reykjavik, Iceland; [3]Institute of Biology, University of Iceland, Reykjavik, Iceland

Hydrogel formulations containing the 1-monoglyceride of capric acid (monocaprin) have been shown to possess potent *in vitro* microbicidal activity against HIV-1, HSV-1, HSV-2, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Since (i) there is no animal model available to study the effect of virucidal agents on vaginal transmission of HIV and (ii) HSV-2 is *in vitro* about as sensitive to the virucidal action of the gel formulations as HIV-1, we evaluated the effect of the hydrogel formulations on intracutaneous and intravaginal infection of mice with HSV-2. When applied as a 20 mM gel on the mouse skin before scarification and subsequent inoculation with HSV-2, complete protection of lesion development and therewith associated mortality was observed. Similarly, intravaginal infection with HSV-2, and therewith associated mortality in mice, was completely prevented when the infection was carried out in the presence of gel containing 20 mM monocaprin. No irritation or toxicity was observed following application of the gel to either the skin or the vaginal mucosa. Hydrogel formulations of monocaprin should thus be pursued for use as vaginal microbicides in the prevention of sexual transmission of enveloped viruses including HIV and HSV.

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The healthcare costs of genital herpes in the US - a pharmacoeconomic model.

Stanberry L[1], Handsfield H[2], Sacks S[3], Peters B[4], Chinn C[5].
[1]Div Infect Dis, Children's Hospital Research Foundation, Univ Cincinnati; [2]Univ Washington and Public Health of Seattle-King County; [3]Viridae Clinical Sciences Inc. and Dept Pharmacol & Therapeutics, Univ British Columbia; [4]St Thomas' Hospital, London; [5]SmithKline Beecham Pharmaceuticals, Harlow, UK

The prevalence of genital HSV infection has increased in the United States over the past two decades, making genital herpes a significant public health problem. To examine the economic impact of genital herpes, we have developed a model designed to estimate the overall health care costs associated with symptomatic infection in the US. Estimates of the annual number of new cases and number of recurrent infections were based on published data. The distribution of cases by virus type and infection type (i.e. where the first symptomatic infection is either an initial infection or a recurrent infection) were based on data from a recent large multicentre study of antiviral treatment for first episode genital herpes. Estimates of health care costs associated with first episode and recurrent genital herpes were derived from a medical claims database. The clinical trial screening data showed that of subjects seeking medical care for an apparent first episode of genital herpes, 31% experienced first episode HSV-1, 45% first episode HSV-2 and 24% first diagnosed recurrence of HSV-2. Using these data, together with estimated probabilities of recurrences in patients with each type of HSV infection, and a minimal estimate of 450,000 new cases being diagnosed in US each year, the total cost of HSV infection within the US in a single year was estimated to be $468.3 million. Taking into account the costs of genital herpes in pregnancy, the total US annual economic burden due to genital herpes rises to $663 million, which, while a substantial sum, is considered a conservative estimate. Clearly, genital herpes is a disease with significant impact on health care resources. How these would be affected by future disease management options will be discussed.

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Genvir, a Sustained-Release Formulation of Acyclovir Given Twice-Daily, is at Least Equivalent To Zovirax®, Given Five Times Daily for the Episodic Treatment of Recurrent Genital Herpes. P. JOLY[1], H. FUDER[2], P. VIVET[3], G. SOULA[3]. [1]*Clinique Dermatologique, Hôpital Charles Nicolle, Rouen, France* [2]*Parexel GmbH, Berlin, Germany,* [3]*Flamel Technologies, Vénissieux., France*

Background: Zovirax® (Glaxo-Wellcome, Greenford, UK) is a safe and efficacious treatment of the recurrences of genital herpes (RGH). However, due to the short residence time of acyclovir in the body, the recommended dosage (200 mg, five times daily) may be inconvenient to the patients and associated with suboptimal adherence. A sustained release formulation of acyclovir, Genvir, has been devised, using the Micropump® microparticle system, to be compatible with a twice-daily dose regimen. **Methods**: This multicenter, multinational, double-blind European trial compared patient-initiated Zovirax® (200 mg, 5 times a day) and Genvir (600 mg, twice a day) in immunocompetent patients with RGH. The primary endpoint was the percentage of patients with complete healing (re-epithelialization) of herpes lesions on Day 5 after treatment initiation. Secondary outcomes included the percentage of patients with new herpes lesions after treatment initiation, the aborted episode rate and the cumulative incidence of herpes symptoms. **Findings**: 424 patients presented with a RGH and were included in the intent-to-treat analysis. The percentages of healed patients at Day 5 were 53.6% and 45.7% for Genvir and Zovirax® respectively (p=0.106, 95% CI: [-0.12%, 15.96%]). New herpes lesions occurred in 4.0% of Genvir-treated patients and 9.1% of Zovirax®-treated patients (p=0.034). The aborted episode rate was 7.2% and 6.1% for Genvir and Zovirax® respectively (NS). The incidence of herpes symptoms was very similar with both drugs. No serious adverse event was observed with either drug and the adverse events were similar with both drugs and in agreement with the well-known very safe profile of acyclovir. **Interpretation**: Genvir has been shown to be at least equivalent to Zovirax® in the episodic treatment of RGH. If confirmed, these results might make Genvir a suitable therapeutic alternative to second generation anti-herpes drugs such as valaciclovir and famciclovir in this indication.

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Acyclovir-Resistant Herpes Simplex Virus in HIV-Infected Individuals: Results from an Ongoing U.S. Surveillance Study.

J. W. Gnann[1], F. M. Clark[1], C. A. Hodges-Savola[2], N.T. Wetherall[2], E. R. Kern[1], and the Task Force on Herpes Simplex Virus Resistance.
[1]University of Alabama at Birmingham, Birmingham, Alabama
[2]ViroMed Laboratories, Minneapolis, Minnesota

Isolates of acyclovir (ACV)-resistant (R) herpes simplex virus (HSV) were recognized soon after ACV became available for clinical use. Virtually all ACV-R HSV isolates are recovered from immunocompromised patients and the vast majority have been HSV-2. Widespread use of ACV and related drugs (valacyclovir, famciclovir, penciclovir, ganciclovir) has raised concerns about the potential for increased frequency of ACV-R. In a prior study conducted in collaboration with the CDC, >2000 anogenital HSV isolates were collected from STD and AIDS clinics and screened for ACV-R. Across the population, < 1% of HSV isolates were ACV-R, but among isolates obtained from patients known to be HIV-seropositive the ACV-R rate was about 5%. This ongoing study is focused on HIV-seropositive individuals as a sentinel population for changes in incidence of ACV-R. Surveillance now includes both orolabial and anogenital lesions. From April-December, 1998, 191 specimens from HIV-seropositive patients with suspected HSV infections were submitted from 9 surveillance sites in the U.S. Cultured lesions were 68% anogenital and 28% orolabial. Eighty-one specimens were culture positive for HSV (42.4%), with 67 HSV-2 (83%) and 14 HSV-1 (17%). The median age of the population was 38 years; the median CD4 count was 143/mm^3. 51% of the population had previously taken antiherpesvirus drugs. *In vitro* testing for ACV-R (defined as EC_{50} >2.0 μg/ml) is complete on 62 of 81 isolates. HSV isolates from 4 patients (6.5%) were ACV-R (median EC_{50} > 30 μg/ml); all were HSV-2 and all were recovered from anogenital lesions. *In vitro* characterization of these isolates is in progress. The median CD4 count for patients with ACV-R isolates was 18/mm^3; all reported prior episodes of anogenital HSV and all had taken antiherpesvirus drugs. The frequency of ACV-R HSV in immunocompetent and immunocompromised patients has remained stable over 15 years, but continued surveillance in both populations is essential to monitor potential increases in incidence.

Poster Session II: Herpesvirus and Respiratory Virus Infections

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Antiadenovirus and Antiherpes Virus Activity of 6-Azacytidine

N. Dyachenko, *S. Rybalko, L. Nosach, **I.Alexeeva, **A. Shalamay

Inst. of Microbiol. & Virol. Nat.Acad.Sci; *Inst. of Epidemiol. & Infect. Dis.; **Inst. Molec.Biol. & Genetics Nat.Acad.Sci., Kiev, Ukraine

It was established that 6-Azacytidine (6-AC), the structural cytidine analogue, inhibits the reproduction of adenoviruses type 1 in cell culture, selectivity index of 6-AC was 125.

It was established that 6-AC inhibits the synthesis of viral DNA, proteins, formation of intranuclear inclusion bodies and synthesis of infective virus, in some concentration it completely caused switch-off adenovirus genome expression. 6-AC activity was revealed against herpes simplex virus of type 1 on the model of meningoencephalitis of mice in prophylactic and medical schemes use, its effectivity was more than reference compound (acyclovir, "KRKA", Slovenia).

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N,N′-bisheteryl Derivatives of Dispirotripiperazine: A Novel Class of Antiherpetic Compounds Blocking Virus Adsorption

M. Schmidtke[1], A. Stelzner[1], V. Makarov[2]

[1]Institute of Virology, Friedrich Schiller University of Jena, Winzerlaer Str. 10, 07745 Jena, Germany, e-mail: i6scmi@pop3.uni-jena.de, [2]State Scientific Center of the Russian Federation NIOPIK, B. Sadovaya str. 1, block 4, 103787 Moscow, Russia

N,N′-bisheteryl derivatives of dispirotripiperazine were examined for cytotoxicity, anti-coxsackievirus B3, anti-influenzavirus A, and anti-herpes simplex virus type 1 (HSV1) activity under in vitro conditions. In result of the primary antiviral screening, some compounds were proved to be very effective inhibitors of the HSV1-induced cytopathic effect (CPE) in GMK cells with therapeutic indices ($TI=CC_{50}/IC_{50}$) > 100. Surprisingly, the potent antiherpetic activity of these compounds could be confirmed in a plaque reduction assay only if the compounds were present during virus adsorption. Using this condition, the dispirotripiperazine derivatives reduced the plaque formation both of HSV1 and HSV2. Additionally, they were active against aciclovir- and foscarnet-resistent HSV1 and HSV2 strains.

Investigations about the mechanism of activity showed that these compounds act not virucidal. Furthermore, plaque reduction was detected both when cells were preincubated with the compounds for 1 h at 37°C and subsequently removed before virus inoculation and when the compounds were present only during virus adsorption (1 h, 37°C). If GMK cells were preincubated for raising periods (2, 4 h at 37°C) with the derivatives of dispirotripiperazine before HSV1 was added, the protecting effect was increased. Taken together, our data indicate that N,N′-bisheteryl derivatives of dispirotripiperazine are very effective in the treatment of herpes virus infections under in vitro conditions and that the mechanism of antiviral activity based on the inhibition of virus binding to the target cells. Currently, we are interested in studies about (i) structure-activity relationships, (ii) the antiviral activity against other members of the herpesviridea, (iii) the benefit of combination therapy, and (iiii) the in vivo efficacy and toxicity.

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An *In Vitro* Assay for Evaluating the Antiviral Activity of HSV Thymidine Kinase Inhibitors

P Wong Kai In, MJ Holland, JA Martin, JH Merrett, DS Pole, NA Roberts, GM Taylor and GJ Thomas
Roche Discovery, Welwyn Garden City, Hertfordshire, UK

An *in vitro* cell culture system, suitable for the assessment of the antiviral potential of HSV-TK inhibitors, has been developed. In this assay, monolayers of 143b cells (a human osteosarcoma [TK-] cell line) are treated with HAT (hypoxanthine, aminopterin and thymidine) for 3 hours prior to and during infection with HSV-2 (333) at a multiplicity of infection of 0.01 pfu/cell. After virus adsorption duplicate cultures are treated with a range of concentrations of TK inhibitors. The treated cultures are then incubated for 18 hours after which the infected cells and supernatants are harvested and the virus yields determined by titration on Vero cell monolayers. The use of the cytostatic agent, aminopterin, is to block the cellular pathway for *de novo* biosynthesis of thymidylate which is required for viral DNA synthesis. The virus, however, can through its own encoded thymidine kinase salvage the exogenously supplied thymidine and sustain its own DNA synthesis and replication. Hypoxanthine is supplied as the preformed purine for the cellular salvage pathway. Studies with HSV-2 (333) comparing the replication of wild-type with a TK-deletion mutant in HAT-treated 143b cells showed that the growth of the mutant was severely impaired resulting in at least a 99% reduction in virus yield relative to the wild-type. Virus yields in 143b cells maintained in normal medium were comparable. It is, therefore, reasonable to conclude that an effective HSV-TK inhibitor would also reduce virus yields in 143b cells treated with HAT by limiting the extent of viral DNA replication. Indeed, we demonstrate that a range of potent HSV TK inhibitors show good antiviral activity in this assay. Furthermore, there is a good correlation between potency against isolated enzyme and antiviral minimum inhibitory concentration (MIC) which is defined as the concentration required to reduce virus yield by >90%.

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Inhibition of the Growth of Herpes Simplex Virus in BS-C-1 Cells by Vaccinia Virus. K. Keywan and E. Katz. Department of Virology, Hebrew University - Hadassah Medical School, Jerusalem, Israel.

The growth of herpes simplex virus type 2 (HSV-2) in BS-C-1 cells, was significantly inhibited by super-infection with vaccinia virus. The inhibition was induced by both the intracellular mature virus (IMV) form of vaccinia virus and extracellular enveloped virus (EEV), which contains an additional external viral membrane. Treatment of vaccinia IMV with the detergents NP-40, Brij-58 or n-octyl-α-D-glucopyranoside abolished its ability to inhibit the growth of HSV-2. However, ultraviolet (U.V.)-irradiation of vaccinia virus, that efficiently inactivated the infectivity of the virus, did not affect its capability to inhibit the growth of HSV-2. The irradiated vaccinia virus was found to adsorb and penetrate into the HSV-infected cells but failed to uncoat its membranes and thus remained morphologically intact within the cells for at least 22 h. As a result of the treatment of HSV infected cells with the irradiated vaccinia virus, the level of HSV DNA synthesis was only partially effected but the synthesis of viral proteins and the formation of virus particles were almost completely blocked.

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Anti - herpes simplex virus (HSV) activities of natural carrageenans in neural cells. M.J. Carlucci*, L.A. Scolaro*, M.D. Noseda**, M. Ciancia**, E.B. Damonte*. *Lab. de Virología and **Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428. Buenos Aires, Argentina.

Carrageenans of diverse structural types (λ, κ/ι, and μ/ν) isolated from the red seaweed *Gigartina skottsbergii* proved to be potent inhibitors of HSV types 1 and 2 in a plaque reduction assay in Vero cells. Due to the neurotropic properties of both viruses, their response to polyanionic compounds was studied in cells of neural origin. The antiherpetic activity of the carrageenans 1T1, 1C1 and 1C3 was evaluated by a yield assay in astrocyte-enriched cultures obtained from neonatal mouse brains. The three carrageenans reduced HSV-1 and HSV-2 production in a dose-dependent manner and the efficacy of virus inhibition was similar to that observed in Vero cells, with IC50 values in the ranges 0.9-3.6 and 0.4-3.2 μg/ml for murine astrocytes and Vero cells, respectively. No cytotoxicity was detected at concentrations up to 1000 μg/ml, indicating high selectivity indices for these compounds. Other HSV-induced alterations of astrocytic properties were also reversed in the presence of the carrageenans. The increase in the expression of glial fibrillary acidic protein (GFAP), an activated astrocyte marker, produced during the course of HSV-1 infection in astrocytes, was reversed in the presence of 1C1 and 1C3. By contrast, 1T1 increased the number of astrocytes expressing GFAP, independently of HSV-1 infection. The level of procoagulant activity, due to the expression of tissue factor, was also down-regulated in treated infected cells. The modulatory action of carrageenans was specific for these activated proteins, because no alteration in the level of total cellular protein synthesis was detected by radioactive labeling. The protective effect of carrageenans in neural tissue was also evaluated in vivo. The intracerebral inoculation of neonatal OF1 mice with HSV-1 and 1T1 reduced significantly the mortality and the level of HSV-1 replication in mouse brain when compared with infected control animals.

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Potent Anti-HSV Effect of HPMPC in Resting Macrophages

E. Balestra, F. Marcuccilli, J. Balzarini*, B. Tavazzi, S. Aquaro, CF. Perno, R. Calió
Dept. of Experimental Medicine University of Rome 'Tor Vergata', Italy and *Rega Institute, Catholic University of Leuven, Belgium

Herpes simplex virus (HSV) in vivo infects cells like neurons, that are characterized by a limited DNA metabolism, far different than that of replicating cells utilized in experimental system. Differentiated M/M supply an optimal model of resting cells infected by HSV-1, that can mimic primary neurons, whose cultivation is quite difficult. Objective of this study was then the assessment of the anti-HSV-1 efficacy and cellular metabolism of (S)-1-[3-hydroxy-2(phosphonylmethoxy)propyl]cytosine (HPMPC), an acyclic nucleoside analog with potent activity against a broad spectrum of herpesviruses. Mature M/M were exposed to various concentrations of HPMPC and then challenged with different MOIs of HSV-1. The assessment of virus production was performed in the supernatants of M/M by plaque formation assay, 48 hours after virus challenge. The results were then compared with those obtained in fibroblastoid VERO cells. HPMPC efficiently inhibited the replication of HSV-1 in M/M, with an EC_{50} in the range of 0.01-0.1μM, independently of the different MOIs used. Such antiviral efficacy in M/M was up to 100 fold greater than that achieved in VERO cells. This greater efficacy in M/M is related to the resting status of M/M. Indeed the endogenous levels of dCTP, the natural counterpart of HPMPC-DP, are about 10 fold lower in M/M compared to replicating VERO cells. Thus, although the phosphorylation of HPMPC in M/M is in the same range of that achieved in VERO cells, the overall ratio HPMPC-DP/dCTP is at least 8 fold greater in M/M. This greater ratio accounts for the increased efficiency of HPMPC in inhibiting HSV-1 DNA-polymerase in M/M, and consequently for the marked anti-HSV effect found in these cells. These results suggest that the study of the anti-HSV activity of HPMPC only in replicating cells may have so far underestimated the real effect achieved in HSV-infected patients, because the compartment of resting cells (such as neurons and macrophages) can be even more sensitive to the antiviral effect of this drug.

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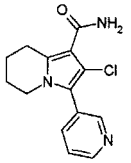
RPR CMV423: a new chemical family, as potent *in vitro* inhibitors of Human Cytomegalovirus : Synthesis, structure-activity relationships and biological data.

C. Nemecek°,G. Andrei*, E. Bacqué°, G. Bashiardes°, A. Bousseau°, E. De Clercq*, N. Dereu°, C. Roy°, R. Snoeck*.
* Rega Institute, Leuven, Belgium.
°Rhône Poulenc , Vitry sur Seine, France.

The human cytomegalovirus (HCMV) belongs to the herpesvirus family and is widely present at a latent stage in the human population. HCMV leads, in immunosupressed patients (AIDS, organ transplants), to a life threatening disease. Current antiviral treatments for HCMV infections target mainly the DNA polymerase without reaching optimal efficacy and leading to serious side-effects. Therefore, there is a need for new potent antiviral agents for HCMV disease acting by a different mechanism.
RPR CMV423 was found by us to be one of the most potent compound ever described against HCMV *in vitro* (IC_{50}= 0.0058 µg/ml Davis lab strain).
RPR CMV423 strongly synergizes with HCMV DNA polymerase inhibitors and presents no *in vitro* cross-resistance with these drugs.
RPR CMV423 is active on an early step of the virus cycle and does not interfere with DNA polymerase.
The synthesis and structure-activity relationships of RPR CMV423 and analogs, a non-nucleoside chemical series, will be presented.



RPR CMV423

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2-Chloro-3-substituted-1,4-naphthoquinone Inhibitors of HCMV Protease: SAR and Mode of Action Studies.

M.J. Slater and P. Ertl. Glaxo Wellcome,
Gunnels Wood Road, Stevenage, SG1 2NY UK.

HCMV protease plays a critical role in capsid assembly and viral maturation and is an attractive target for antiviral chemotherapy. HCMV protease is a serine protease, the crystal structure of which has recently been reported, revealing a novel protein fold and novel catalytic machinery involving Ser-132 and two histidine residues (His-63, His-157). From a screening exercise to discover novel inhibitor templates, we identified 2-chloro-3-(2,6-dioxo-4,4-dimethyl-cyclohexyl)-1,4-naphthoquinone as a moderate, time-dependent, enzyme inhibitor (IC_{50}=150µM). Further screening around this template identified inhibitors such as 2-chloro-3-(1-acetyl-2-oxopropyl)-1,4-naphthoquinone with submicromolar potencies. The covalent interaction between 2-chloro-3-(1-acetyl-2-oxopropyl)-1,4-naphthoquinone and the enzyme was characterised by ES mass spectroscopy and tryptic peptide mapping.

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Comparison of the *in vitro* antiviral activities of 1263W94, ganciclovir, foscarnet and cidofovir for human cytomegalovirus (HCMV) clinical isolates as determined by flow cytometry and plaque reduction assays.

JJ MCSHARRY[1*], NS LURAIN[2], M DAVIS[3], CL TALARICO[3], KK BIRON[3], AND CS CRUMPACKER[4]. [1]Albany Medical Ctr, Albany, NY, USA, [2]Rush Medical Coll, Chicago, IL., USA, [3]GlaxoWellcome Inc., Research Triangle Park, NC, USA, and [4]Beth Israel Deaconess Medical Ctr, Boston, MA, USA.

A flow cytometry drug susceptibility assay was used to determine the IC_{50} values of HCMV clinical isolates for 1263W94, ganciclovir, foscarnet, and cidofovir. The results of the flow cytometry assay were compared with those obtained with the standard plaque reduction assay (PRA). For the flow cytometry assay, HFF cell monolayers were infected at an MOI of 0.1 infected cell/cell in the presence of various concentrations of drug (1263W94, 0 to 2.5 µM; ganciclovir, 0 to 96 µM; foscarnet, 0 to 800 µM; and cidofovir, 0 to 8 µM). After 144 hr of incubation, the cells were harvested, permeabilized, treated with an FITC-labeled monoclonal antibody to an HCMV late antigen (MAB8127, Chemicon International, Inc. Temecula, CA), and the percentage of antigen positive cells was determined by flow cytometry. The average IC_{50} values by flow cytometry and the PRA for drug sensitive clinical isolates were 0.366 ±0.17 and 0.347 ± 0.134 µM 1263W94; 4.12 ±1.99 and 2.54 ± 1.21 µM ganciclovir; 191.18 ± 82.49 and 139.05 ± 59.69 µM foscarnet; and 0.702 ± 0.29 and 0.890 ± 0.32 µM cidofovir, respectively. With the exception of cidofovir, the IC_{50} values obtained by flow cytometry were slightly higher than those obtained with the PRA. Both assays gave IC_{50} values that were highly comparable for clinical isolates that are susceptible to the respective drugs.

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A Novel DNA Hybridization Assay for the Evaluation of Antiviral Activity Against Human Herpesvirus Type 6 (HHV-6)

L. Naesens*, L. De Bolle, J. Neyts and E. De Clercq
Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

The disease pattern that has been associated with human herpesvirus 6 (HHV-6) includes exanthema subitum in children and lymphoproliferative and neurological disorders in immunodeficient individuals (i.e., bone marrow transplant recipients and AIDS patients). Data on the sensitivity of HHV-6 to antiviral compounds have been rather limited, the main drawback being the lack of a uniform *in vitro* assay that is both convenient and reliable. We have now developed a DNA hybridization technique, that is based on the non-radioactive detection of HHV-6 DNA using dot blot and hybridization with an HHV-6-specific probe that recognizes both A and B variants of HHV-6. We used this assay to determine the inhibitory effect of several antiherpetic compounds on HHV-6 (strain GS) replication in human T-lymphocyte HSB-2 cells. The concentration that produced 50% reduction in viral DNA band intensity (EC_{50}) was: 33 µg/ml (acyclovir); 6.8 µg/ml (ganciclovir); 71 µg/ml (penciclovir); 2.8 µg/ml (lobucavir); 2 µg/ml (foscarnet) and 2.1 µg/ml (cidofovir). These EC_{50} values were similar to those obtained by a microscopical cytopathicity assay. The relatively weak antiviral activity and low selectivity index obtained for acyclovir, ganciclovir, penciclovir and lobucavir support the hypothesis that HHV-6 lacks a functional thymidine kinase (as encoded by HSV) or phosphotransferase (as encoded by CMV). This is consistent with our metabolism studies, which indicated that the phosphorylation of radiolabeled acyclovir in HSB-2 cells is inefficient and similar for uninfected and HHV-6-infected cells. In addition, we found no evidence for upregulation of cellular kinases during HHV-6 replication in lymphocytic cells.

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The Immunosuppressive Agent Mycophenolate Mofetil Markedly Potentiates the Activity of Lobucavir against Different Herpesviruses

J. NEYTS and E. DE CLERCQ
Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Mycophenolate mofetil (MMF) has been approved as an immunosuppressive agent in kidney transplant recipients and may thus be used concomitantly with antiherpetic agents, the latter for the treatment of intercurrent herpesvirus infections. The parent compound of MMF, mycophenolic acid (MPA) is a potent inhibitor of IMP dehydrogenase and causes depletion of the intracellular GTP pool levels. Lobucavir {1R(1alpha,2beta,3alpha)]-9-[2,3-bis(hy-droxymethyl)cyclobutyl]guanine (LBV)} is a novel antiviral agent with activity against ganciclovir-resistant cytomegalovirus (CMV) strains, that is in phase II clinical trials for the treatment of CMV infections. LBV triphosphate (LBV-TP) inhibits the viral DNA polymerase competitively with dGTP. When combined with LBV, MPA (at concentrations ranging from 0.25 to 10 μg/ml, which are readily attainable in human plasma) markedly potentiated the antiviral activity of LBV against HSV-1 and HSV-2, that is a 10- to 100-fold decrease in EC_{50}. The EC_{50} of LBV against TK^- HSV-1 decreased even 1400-fold upon combination with MPA. MPA by itself had little or no effect on the replication of HSV-1, HSV-2 or TK^- HSV-1. In addition, MPA and MMF resulted in a marked increase in the anti-CMV activity of LBV (FIC_{min}: 0.24 and 0.26, respectively). Exogenously added guanosine reversed the potentiating effect of MPA on the antiviral activity of LBV, which indicates that this potentiating effect resulted from a depletion of the endogenous dGTP pools, thus facilitating the inhibitory action of the LBV-TP on the viral DNA polymerase. Ribavirin, another inhibitor of IMP dehydrogenase, also caused a marked enhancement of the antiviral activity of LBV against HSV-1 (12-fold), HSV-2 (20-fold) and TK^- HSV-1 (25-fold). The observed drug interaction may have important implications when using LBV in the treatment of intercurrent herpesvirus infections in transplant recipients under MMF therapy.

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Hydroxyurea Enhances the Antiviral Activity of Nucleoside Analogs with Anti-Herpetic Activity

J. NEYTS and E. DE CLERCQ
Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Hydroxyurea has been shown to potentiate the anti-HIV activity of several 2',3'-dideoxynucleoside analogs. We have now studied the effect of hydroxyurea on the antiherpes virus activity of nucleoside and nucleoside phosphonate analogs. At concentrations ranging from 25-250 μg/ml hydroxyurea was found to potentiate by 2- to 60-fold the anti-HSV-1 and anti-HSV-2 activity of ganciclovir, acyclovir, penciclovir, lobucavir, H2G, brivudin, cidofovir and adefovir. Hydroxyurea itself had, at the concentrations used, little or no effect on viral replication, as assessed by means of virus yield reduction assays. Hydroxyurea also markedly increased the antiviral activity of ganciclovir, acyclovir, penciclovir, lobucavir and H2G, compounds that depend for their activation on a virus-encoded thymidine kinase (TK), against TK-deficient (TK^-) strains of HSV-1. In fact, in combination with hydroxyurea the 50% inhibitory (effective) concentration (EC_{50}) of these compounds for TK^- HSV-1 decreased 20 to ≥ 100 μg/ml to 1 to 5 μg/ml. Combination studies with natural nucleosides revealed that the potentiating effect of hydroxyurea mainly stems from a depletion of the intracellular dNTP pools, thus favoring the triphosphates of the nucleoside analogues or the diphosphates of the nucleoside phosphonate analogues in their competition with the natural nucleotides at the viral DNA polymerase level.

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The pathogenesis of HSV-1 and HSV-2 penciclovir or ayclovir-selected TK mutants in the zosteriform murine infection model.

A.R. Awan[1], T.H. Bacon[2], R.T. Sarisky[3], J.J. Leary[3], D. Sutton[3], H.J. Field[1].
[1]Centre for Veterinary Science, University of Cambridge, Madingley Road, Cambridge, CB3 0ES. SmithKline Beecham: [2]Consumer Healthcare, Surrey KT13 0DE, UK. [3]Pharmaceuticals, 1250, S. Collegeville Rd, Collegeville, PA. 19426, USA.

The pathogenesis of wild-type viruses, HSV-1(SC16) and HSV-2(SB5) and several penciclovir (PCV) or acyclovir (ACV)-selected resistant mutants were compared in the zosteriform infection model. Mice were infected by scarification at the lateroventral line of the neck, clinical signs recorded and levels of infectious virus monitored in the target organs. For HSV-1(SC16), lesions first appeared on the ipsilateral ear pinna on day 5 or 6 p.i. For HSV-2(SB), the clinical signs were less severe and the zosteriform spread to the ear was delayed until day 8 or 9 p.i. The resistant HSV mutants were selected by passage in MRC-5 cells in the presence of PCV (3 mutants) or ACV (1 mutant). The mutants were TK^{-ve} in BHK-BU cells (<1% wild-type enzyme activity), and by $^{[125]}$I-iododeoxycytidine autoradiography, no plaques were positive (<0.01%). The characterisation was confirmed by sequencing the TK gene and western blot analysis; three mutants have truncated TK and one HSV-1 mutant appears to express full-length TK product. The two HSV-1 mutants replicated briefly to high titre at the local site of inoculation, but infection waned faster than wild-type. One of the two HSV-2 mutants behaved similarly to wild-type at the local site. At the secondary site (ear pinna), the titres of wild-type virus reached 10^6 and 10^5 pfu/ear for HSV-1 and HSV-2 respectively. In contrast, no clinical signs or infectious virus were detected in the ear pinnae of mice inoculated with any of the four mutants. These data suggest that PCV-selected TK^{-ve} viruses have markedly attenuated pathogenicity consistent with previous reports for ACV-selected TK^{-ve} mutants. Further mice were inoculated in the neck and valaciclovir or famciclovir supplied in the drinking water (approx. 160 mg/Kg/day) from 24h p.i. to day 10. Both compounds were very effective; reducing clinical signs and virus replication at the local site and suppressing zosteriform spread to the ear. The sensitivity of the resistant mutants to chemotherapy in this model is currently under investigation.

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A mouse model which employs zosteriform spread of HSV-1 in the presence of adoptive transfer of immunity and its application for the study of antiviral therapy.

P.M. Calvi[1], J. Harmenberg[2], R. Bujdoso[1], A.R. Awan[1], T. Liden[2], H.J. Field[1]
[1]Centre for Veterinary Science, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK.
[2]Medivir AB, Lunastigen 7, S-141 44 Huddinge, Sweden.

The aim of this study is to obtain a reproducible murine model for recurrent cutaneous HSV-1 in humans, that occurs in presence of an established immune response, and is suitable for investigating chemotherapy. We have therefore adapted the zosteriform HSV-1 infection model by the adoptive transfer of immunity from primed donor mice. Two groups of donor Balb/c mice were prepared by inoculation of 10^5 or 10^6 p.f.u./mouse of HSV-1 (SC16), into the skin of both ears. The swollen draining cervical lymph nodes were removed 7 days post infection and a suspensions of lymph node cells obtained. 10^6 cells/mouse were transfused into the tail vein of four groups of syngeneic mice, infected two days previously by scarification and application of 10^8 or 10^7 p.f.u./ml of HSV-1(SC16) at the right latero-ventral line of the neck. Control groups received mock infection or no immune cells. The clinical signs in the recipient mice, particularly those associated with zosteriform virus spread to the ipsilateral ear pinna, were recorded daily and infectious virus titres were measured in the skin and neural tissues. The results clearly demonstrated that transfer of immune cells exacerbate the clinical signs of infection. But the model was found to be sensitive to virus inoculum dose and to the dose of virus used to infect the donor mice. The data demonstrate the delicate balance between the level of infectious virus and the intensity of the inflammatory response and associated clinical signs. The critical components of the transferred immune cells are currently under investigation. The model was used to study the effects of the anti-inflammatory drug hydrocortisone on the pathogenesis of infection and the potential for hydrocortisone in combination with acyclovir to control virus growth and the up-regulated clinical signs of disease produced in the ATI model.

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Virulency and reactivation of various TK-deficient mutants of HSV-1 in mouse infection models. C-K. Lee, J.H. Kim, PK Bae and H.S. Kim. Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology, Taejon 305-600, Korea

To investigate influence of viral TK gene on in vivo virulency and reactivation, analyzed were TK DNA sequences and western profiles of AR1 ~ AR9, the laboratory derived TK-deficient acyclovir-resistant mutants of HSV-1 strain F and three mouse infection models were used. All of them except AR5 showed at least one amino acid change in ORF and AR3 and AR8 frame shift mutation causing extanded or premature translation, respectively. AR5 and AR9 showed base changes in 5'-NCR. To check virulency the infected female BALB/c mice were coded and daily clinical symptoms and their body weight were measured for at least 2 weeks. At 30 day p.i. the survived mice were sacrificed and their tissues were isolated: trigerminal nerves from the intranasally or the intracerebrally and sensory ganglia from the cutaneously inoculated. Reactivation of the latenly infected was monitored by using Vero cell culture system. AR2 and AR9 were highly virulent, as high as strain F and AR1 was the least virulent in all of 3 infection models. F, AR2, AR4, AR5, AR6 and AR9 showed secondary lesions on the skin in zosteri model. LD_{50} of each virus - F and AR1 ~ AR9 - in intracerebral infection model as following: 1,200, >2,575,000, <195, 12,000, 198,000, 42,000, 227,000, 22,000, 20,000, and 125 PFU/mouse, respectively. Reactivation was observed in the mice infected with F, AR2, AR4, AR5, AR6 and AR9.

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A Mechanism for Famciclovir Disabling the Establishment of Herpesvirus Latency: A Hypothesis.

R. A. Vere Hodge
Vere Hodge Antivirals Ltd. Leigh, Reigate, UK.

The effects of famciclovir and valaciclovir on the establishment of latency by herpes simplex viruses 1 and 2 have been examined in a mouse model by Thackray and Field. Famciclovir, but not valaciclovir, caused a dramatic reduction in the reactivation potential of ganglion explants. Although the initial infection of the ganglia in mice is fast, probably within 24 h, the process to establish fully competent latent infection in ganglia takes more than 5 days. This was proved by famciclovir treatment having an effect when dosing was started 5 days after infection. However famciclovir treatment did not prevent viral DNA becoming latent. A quantification of HSV latency at the single-cell level (reported by Sawtell) showed that individual neuronal cells may have between 1 and over 1000 HSV genomes. The quantity of latent virus DNA correlates with, and may be a major determinant of, the rates of reactivation of HSV. This presentation proposes a mechanism for the action of famciclovir in disabling the establishment of herpesvirus latency. The affinity of penciclovir for the viral thymidine kinase inhibits the relatively slow process converting a newly-infected neurone into one with a competent latent infection. Once activated, the immune system prevents further development of immature latently infected neurones into competent latent infections. Thus famciclovir therapy, during the primary infection, is able to prevent the establishment of competent latent infection. As aciclovir has much lower inhibitory activity for viral thymidine kinase than does penciclovir, this mechanism suggests that the lack of effect of aciclovir to reduce recurrences in patients should not be taken as predictive for famciclovir. A clinical trial is ongoing.

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Use of the SCID-hu Retinal Implant Model for Determining the Efficacy of Antiviral Therapies Against Human Cytomegalovirus Infections. D.J. Bidanset, R.J. Rybak, C.B. Hartline, and E.R. Kern. The University of Alabama School of Medicine. Birmingham, AL, USA

Human Cytomegalovirus (HCMV) infections can cause a wide range of clinical manifestations in both the normal and immunocompromised host. Currently, there are few animal models to study HCMV as virus replication is largely limited to human cells. In these studies, we utilized fetal human retinal tissue implanted in the anterior chamber of the SCID mouse eye and inoculated 2-8 weeks later with 5000-10,000 pfu of HCMV. At various times after infection, animals were sacrificed, eyes removed and homogenized, and HCMV titers quantified by plaque assay. The initial experimental results indicated that there was a correlation between the amount of time an implant is allowed to grow and the virus titers obtained. An observed 3-10 fold increase in viral titer from 4-8 wk old implants suggested that permitting the implant to grow larger increases HCMV titer in the implants. In the next series of experiments, 9-(2-phosphonylmethoxy-ethyl)adenine (PMEA), the oral pro-drug, (bis) POM PMEA , and 2-bromo-5,6-dichloro-1-B-D-ribofuranosyl benzimidazole (BDCRB) were examined for efficacy against HCMV replication in the implants. PMEA administered i.p. at 10 or 30 mg/kg once daily for 28 days was ineffective in reducing HCMV titer in the retinal implants. (Bis) POM PMEA was administered orally at 33 or 100 mg/kg twice daily for 2 weeks followed by once daily for an additional 2 weeks. The results indicate that despite some drug toxicity, 100 mg/kg (bis) POM PMEA was as effective as 45 mg/kg ganciclovir in reducing viral titers in the retinal implant. Finally, BDCRB administered i.p. at 50 mg/kg once daily for 4 weeks or at 25 mg/kg twice daily for 1 week followed by once daily for 3 weeks was examined. The results indicated that at 50 mg/kg, BDCRB reduced viral titers in the retinal implants approximately 5-fold by day 21 and 2-fold by day 28. BDCRB administered at 25 mg/kg was ineffective in reducing viral titers and neither concentration of BDCRB was as effective as 45 mg/kg ganciclovir. These data indicate that the SCID-hu retinal implant model can be useful for determining antiviral activity against HCMV in vivo and may be predictive for efficacy in humans.

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Characterization of Acyclovir-Resistant Herpes Simplex Virus Collected by the Task Force on Herpesvirus Resistance. K.K. Biron[1], M. G. Davis[1], W. K. Lawrence[1], E. R. Kern[2] and the Task Force on Herpesvirus Resistance, [1]Glaxo Wellcome, Inc. Research Triangle Park, NC, USA, [2]University of Alabama at Birmingham, Birmingham, AL, USA.

In an effort to monitor the incidence of acyclovir-resistant (ACV^r) HSV, the Task Force on Herpesvirus Resistance has established a surveillance program. To date >2500 HSV clinical isolates collected from urogential lesions have been screened with sixteen HSV2 isolates identified as ACV^r (IC_{50}>2 μg/ml by plaque reduction assay). Of the sixteen, fifteen were from immunocompromised individuals and one was from an immunocompetent individual. Further characterization of these clinical isolates included cross resistance studies with penciclovir, ganciclovir, cidofovir and foscarnet, and thymidine kinase (Tk) functional studies using ^{14}C-thymidine and ^{125}I-iodo-2'-deoxycytidine plaque autoradiographies. A virulence assay was done with the original (parental) isolates by intracerebral inoculation of young mice to assess the level of pathogenicity. All isolates were pathogenic in mice, indicating the presence of wild type (wt), although some Tk-deficient subpopulations were detected by plaque autoradiography. Homogeneous ACV^r virus populations were isolated by plaque purification. DNA sequencing of the Tk open reading frame was performed to determine the ACV^r -associated genotype. Of fourteen completed sequences, eleven had single nucleotide insertions or deletions (compared to wt) that led to a predicted protein truncation. Eight of these were located within homopolymeric regions of seven guanines or six cytosines, previously referred to as 'G-string' and 'C-cord', respectively. Two samples had nucleotide substitutions leading to predicted amino acid changes in the Tk protein. These viruses had an altered susceptibility (Tka) phenotype, in which both ^{14}C-thymidine and ^{125}I-iododeoxycytidine were incorporated into the nascent viral DNA, but the incorporation was greatly reduced as compared to wt HSV. For one isolate, the parental virus had a wt Tk, but the virus was resistant by plaque reduction to ACV, penciclovir and phosphonoformic acid, yet sensitive to ganciclovir. Virulence in mice was poor. This phenotype is consistent with a mutation in the HSV polymerase gene. This set of well-characterized resistant isolates will serve as a reference collection for future antiviral studies.

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Phenotypic characterisation of a recombinant TK deficient HSV variant and comparison with a cloned clinical HSV isolate with the same TK sequence.

P Collins, W Harris, W Snowden, M Sowa, R Fenton and G Darby; Glaxo Wellcome R&D, Medicines Research Centre, Stevenage, Herts. UK. and the Task Force on Herpes Simplex Virus Resistance.

We previously reported a cloned aciclovir resistant thymidine kinase (TK) deficient variant of HSV type 1 that demonstrated unexpected neurovirulence in mice. Sequence analysis of the TK gene of two representative double plaque purified clones (4A and 4B) derived from this variant revealed a 2G insertion in the 'G-string' motif, resulting in a predicted truncated protein of 182 amino acids. This was confirmed by Western blot analysis. In a mouse model of HSV infection clones 4A and 4B were shown to establish latent infections and demonstrated neuronal spread via the cervical ganglia to a secondary site of infection. These data strongly suggest a function that compensates for the loss of TK activity. However, to eliminate the possibility that the 2G insertion in TK was responsible for the observed phenotype we constructed a variant of SC16, a well characterised strain of HSV type 1, with the same 2G insertion in the 'G-string' motif. In mice this variant, HXT22G, could not be reactivated from a latent infection, furthermore it failed to demonstrate neuronal spread to secondary sites of infection. These characteristics are those normally associated with a TK deficient phenotype. The data with the recombinant virus HXT22G thus further support the hypothesis that strains 4A and 4B possess an, as yet unknown, mechanism to compensate for the loss of TK activity.

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DNA Polymerase Activity of Ganciclovir-Resistant Human Cytomegalovirus

Eizuru Y, Kariya M, Mori S
Division of Persistent & Oncogenic Viruses,
Center for Chronic Viral Disases, Faculty of Medicine,
Kagoshima University, Kagoshima City, 890-8520, Japan

We isolated ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) which showed cross-resistance to cidofovir and had the point mutation in the DNA polymerase gene, resulting in amino acid substitution at codon 501 from Leu to Phe. The DNA polymerases were partially purified from the GCV-resistant HCMV-infected cells and GCV - sensitive HCMV-infected cells, respectively. Then, the activities of DNA polymerase and 3'-5' exonuclease were compared between them. Optimal KCl concentration for DNA polymerase activity was lower in GCV-resistant virus than that in GCV-sensitive one, though the optimal concentration for 3'-5' exonuclease activity was the same. Vmax and Km for DNA template in both polymerase and 3'-5' exonuclease activities were lower in GCV-resistant virus than those in GCV-sensitive virus. The Km for substrate dNTP in polymerase activity was higher in GCV-resistant virus than that in GCV-sensitive virus. In the presence of noncompetitive inhibitor PAA, there was significant difference in 3'-5' exonuclease activities between them, that is, the activity of GCV-resistant virus was more resistant to PAA, though no significant difference was observed in polymerase activities. This phenomenon may show some relation with the rapid appearance of PFA-resistant virus after the PFA rescue therapy.

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Isolation and Characterization of Foscarnet-Resistant Human Cytomegalovirus.

S. Mori and Y. Eizuru
Division of Persistent and Oncogenic Viruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Kagoshima, Japan

To elucidate the mechanism of drug-resistance of human cytomegalovirus (HCMV), mutants were selected in human embryonic lung fibroblasts (HEL) in the presence of phoshonoacetic acid (PAA). Plaque-purified mutants, strain C2 (derived from strain 93-1R) and C72 (derived from strain 91-7S), exhibited 6.5- and 5-fold more resistance to PAA than their parental strains, respectively. Strain C2 and C72 also showed 7.4- and 10.8-fold more resistance to foscarnet (PFA), respectively. The single strand conformation polymorphism (SSCP) and sequencing analysis of the viral DNA polymerase gene revealed that strain C2 and C72 had single amino acid changes from threonine to alanine at residue 700 in conserved region II and alanine to valine at residue 987 in region V, respectively. Baldanti et al.(1996) reported that one foscarnet-resistant clinical isolate had a mutation identical to strain C2 (Thr 700 Ala). Therefore, codon 700 in region II may be one of the mutational hot spots to foscarnet. Sullivan et al. (1993) reported that a recombinant virus, which has an amino acid change from alanine to glycine at residue 987, showed resistance to ganciclovir (GCV). This recombinant virus was sensitive to PFA. In our experiment, strain C72, which has an amino acid change from alanine to valine at the same residue, exhibited resistance to PFA, but sensitive to GCV. Introduction of mutation into the amino acid residue 987 of strain 91-7S by the site-directed mutagenesis is required to verify this phenomenon.

Baldanti,F. et al., J. Virol. (1996) 70: 1390-95,
Sullivan,V. et al., Antimicrob. Agents. Chemother. (1993) 37: 19-25

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RPR111423, A Novel Inhibitor of HCMV Replication is Orally Bioavailable and Biologically Active in Body Fluids

G. ANDREI,[1] R. A. BUGNICOURT,[2] SNOECK,[1] T. DECELLE,[2] C. NEMECEK,[2] E. DE CLERCQ[1] and C. ROY[2]
[1]Rega Institute for Medical Research, K.U.Leuven, B-3000 Leuven, Belgium; [2]Rhône-Poulenc Rorer, Vitry/Seine, France

RPR111423 and its derivatives belong to a novel chemical family displaying potent and selective activity against human cytomegalovirus (HCMV). These compounds act by an entirely novel mechanism of action. Although RPR111423 is active *in vitro* against HCMV replication at an IC_{50} of about 6 ng/ml, as has been confirmed with a wide range of HCMV strains, the compound proved inactive against rat and murine CMV *in vitro*. Thus, it has not been possible to assess the *in vivo* efficacy of the compound in murine or rat CMV models. An *ex vivo* study was performed to evaluate the anti-HCMV activity in plasma at different times after oral administration of RPR111423 to rats. RPR111423 (20 mg/kg), as a suspension in methylcellulose, was administered orally to rats. Plasma samples were collected at different times following drug administration, and serial dilutions of plasma were tested *in vitro* for their activity against HCMV by a plaque reduction assay in human embryonic lung (HEL) cells. In this assay, plasma had to be diluted at least 100-fold to avoid a direct cytotoxic effect of plasma on HEL cells. Antiviral activity of the plasma samples was compared to that of the compound added directly to the cell culture. The results indicated that one hour after oral administration of RPR111423 to rats, a strong antiviral activity appeared in the plasma. The observed activity was equivalent to 3700 times the concentration of RPR111423 required to inhibit virus-induced cytopathicity by 50%. With this bio-assay, antiviral activity could still be detected 4 hours after oral drug administration, suggesting that RPR111423 is orally bioavailable in rats and biologically active in body fluids such as rat plasma.

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Effects of Ganciclovir, Penciclovir and Acyclovir on Apoptosis

MM Shaw[1], PA Watts[2] & HJ Field[1]
[1]Centre for Veterinary Science, Cambridge University, Madingley Road, Cambridge, UK.
[2]Glaxo-Wellcome Research and Development, Stevenage, UK.

BHK cells transformed with the HSV-1 thymidine kinase (TK) gene were treated with increasing concentrations of ganciclovir (GCV), penciclovir (PCV) and acyclovir (ACV) for up to four days. Gel electrophoresis of DNA extracted from GCV- and PCV- (but not ACV-) treated cells showed DNA laddering effects, typical of apoptosis, which appeared to be time and dose dependent. To investigate whether ACV would produce similar effects on prolonged treatment, exposure to ACV was extended to day 7 but no evidence of apoptosis was observed. FACS analysis of Annexin V-labelled drug-treated cells after 3 days exposure to 20uM compound revealed a strong positive peak for GCV-treated cells and a positive peak for PCV-treated cells. However, the effect remained at background for ACV. The results were substantiated using the TUNEL assay. Similar effects were observed when non-differentiated, HSVTK-expressing, rat phaeochromocytoma cells (PC12) were exposed to the compounds. Reverse transcriptase PCR indicated upregulation of GADD45 transcription in GCV- and PCV-treated HSVTK+ BHK cells on day 2 of treatment, with a lesser degree of upregulation in ACV-treated and non-treated cells, suggesting a possible role for p53 in the drug-induced effect. The use of Caspase inhibitors in the above system allowed further speculation on the pathway of GCV/PCV-induced apoptosis.

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DISTRIBUTION TO THE SKIN OF PENCICLOVIR AFTER ORAL FAMCICLOVIR ADMINISTRATION IN HEALTHY VOLUNTEERS, COMPARISON BETWEEN THE SUCTION BLISTER TECHNIQUE AND MICRODIALYSIS.

N. Borg and L. Ståhle, Dept. Clinical Pharmacology, Huddinge University Hospital, SE-141 86 HUDDINGE, Sweden.

Penciclovir is a drug active against herpes simplex viruses located in the basal layer of epidermis. The aim of this study was to compare suction blister technique and microdialysis in order to measure the penciclovir concentration in the skin after a single dose (250 mg) of its prodrug, famciclovir. Suction blister fluid, microdialysate and plasma have been sampled from eleven healthy volunteers for 5 hours after famciclovir administration. Both suction blister technique and microdialysis showed that penciclovir reaches the skin in the concentrations expected to inhibit herpes virus replication. Both in suction blister fluid and in microdialysate the C_{max} has been observed later than in plasma. The microdialysis concentration was influenced by skin temperature and adrenaline-mediated vasoconstriction. Also microdialysis recoveries of penciclovir were studied with respect to the flow-rate of perfusion medium through the microdialysis probe. Microdialysis and suction blister technique can be used to study the time-concentration profile of penciclovir in the skin and microdialysis allows a continuous sampling of the drug during a long time after administration.

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Evaluation of Natural Compounds as Topical Microbicides Against Herpes Virus Type 2: In Vitro and In Vivo Activity. K. Z. Bourne, N. Bourne, S. F. Reising, and L. R. Stanberry. Division of Infectious Diseases, Children's Hospital Research Foundation, Cincinnati, Ohio, USA.

The increased incidence of sexually transmitted diseases including genital herpes necessitates the development of topical microbicides with broad antimicrobial activity. We examined the in vitro and in vivo anti-HSV-2 activity of 19 naturally occurring compounds, which have been reported to have antimicrobial activity. ED50 values were determined in vitro using HeLa cells and HSV-2 strain MS in a modified plaque reduction assay. Values ranged from 0.0039mg/ml for hypericin to 16mg/ml for borneol. In vivo activity of those compounds with good in vitro activity was evaluated using a mouse model of genital herpes. Swiss Webster mice primed with medroxyprogesterone were intravaginally administered 15µl of the test microbicide 20 seconds prior to intravaginal challenge with 4 log 10 PFU of HSV-2 strain 186. With this model <10% of placebo treated mice survive the infection. Of those compounds tested in vivo, carrageenan iota type V, limonene, thymol, cinnamon oil, safrole, tea tree oil, linalool, origanum oil, hypericin and citral were weakly protective with survival rates of 13%, 18.75%, 18.75%, 26.7%, 31.25%, 31.25%, 37.5%, 37.5%, 40% and 43.75% respectively. Marginal protection was afforded by caffeic acid (50% survival), carrageenan lamda type IV (53% survival), cineole (50% survival), and geraniol (50% survival). The best protection was seen with curcumin and eugenol with survival rates of 75% and 87.5% respectively. The in vitro and in vivo anti-HSV-2 activity of curcumin and eugenol suggest that they should be further examined to determine their activity against other STD pathogens.

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Single dose Doxovir™ Reduces the Incidence of Genital Herpes *In Vivo* When Used as a Pre- or Post-exposure Topical Microbicide. N.Bourne and L.R. Stanberry. Division of Infectious Diseases, Childrens Hospital Research Foundation, Cincinnati OH.

Topical microbicides, compounds which can be applied intravaginally, represent a female-initiated approach to the control of sexually transmitted diseases. We have evaluated the organometallic compound Doxovir™ (originally developed for the topical treatment of herpes keratitis) *in vivo* as a microbicide against genital HSV-2 infection. Swiss Webster mice were administered 15µl of 2.0% or 0.5% Doxovir™ solution or saline intravaginally 20 seconds before challenge with HSV-2. All saline control animals became infected (as defined by culture of virus from vaginal swabs collected on the first 2 days post challenge) and 13/15 developed disease. In contrast, none of the animals that received Doxovir™ at either concentration developed disease and all were uninfected by virus culture (each $p<0.01$ vs control). We next showed that when the Doxovir™ concentration was reduced to 0.1% all animals were still protected against infection, and, that even at 0.01% the incidence of infection was reduced by 50% compared to controls. The effect of time of administration was also examined. A single dose of a 2% solution administered 5 minutes before virus challenge provided complete protection against infection and even when administered 60 minutes before challenge there was a 50% decrease in the incidence of infection compared to controls. Further, a single Doxovir™ treatment 6 hours post challenge also produced a 50% reduction in the incidence of infection suggesting that the compound has potential for post-exposure prophylaxis. In a preliminary study we have also shown that Doxovir™ is protective in a second animal species. When female Hartley guinea pigs received 200µl of a 2% solution 20 seconds prior to virus challenge the incidence of primary genital skin disease was significantly reduced compared to saline controls (1/12 vs 9/12; $p<0.005$). Further studies to fully define Doxovir™ as a topical microbicide are planned.

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Microbicidal Effects of CTC-96 *in vitro*. By I. Winicov*, O. Trochev†, A., Pinter†, C.C. Stewart*, and R.M. Burger*†. *Redox Pharmaceutical Corp., Greenvale, NY, U.S.A., and †Public Health Research Institute, New York, NY, U.S.A.

In animal models, the cobalt-Schiff base complex CTC-96 (Doxovir™) prevents infection with genital herpes; it also rapidly cures ocular herpes infections. To investigate the loci of its prophylactic actions we have initiated in vitro studies with the following results. 1) CTC-96 directly inactivates HSV-1, as well as other microbes, including HIV. Treatment of HSV-1 with 100 µg/ml CTC-96 for 30 min caused > 95% reduction in plaque forming activity. CTC-96 inactivated HIV with IC_{50} = 73 µg/ml and IC_{90} = 450 µg/ml. 2) When added to cells pre-infected with HSV-1, CTC-96 reduced plaque formation with an IC_{50} of ~ 20 µg/ml. However, exposure to CTC-96 for 48 h after virus absorption was less effective than 1 h exposure during adsorption, at each concentration tested. 3) Cells treated with CTC-96 prior to challenge with HSV-1 were also protected. This protection was time and concentration-dependent (IC_{50} ~ 300 µg/ml), and reversible. The onset of protection ($t_{½}$ ~ 15 min) was much more rapid than its loss following CTC-96 removal ($t_{½}$ ~ 10 h), which may reflect either prolonged retention of CTC-96 or of its effects. The anti-viral potency of CTC-96 against HSV-1 plaque formation depends on the viral or host target and the time of administration, being most potent when added to virus prior to or during absorption, less so when added to already infected cells, and still less (but effective) when used to pre-treat uninfected cells. These results also suggest that the prophylactic effects of CTC-96 may involve more than one locus. In addition to directly inactivating HSV-1 and HIV-1, CTC96 may alter the host to render it less hospitable to HSV-1.

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Carrageenan Based Formulations for Preventing infection by HSV-2 D. Phillips, V. Zacharopoulos and R. Maguire, The Population Council, 1230 York Avenue, New York, NY 10021 USA

In vitro and in vivo evidence suggests that Nonoxynol-9 (N9) and the sulfated polysaccharides carrageenan may be effective as active ingredients in topical microbicides. We compared vaginal formulations of carrageenan with and without N9 with OTC spermicides for efficacy in protecting mice from infection following vaginal inoculation with herpes simplex virus 2 (HSV-2). Test formulations were 1) 2% carrageenan + 1% carbopol, 2) 3% carrageenan, 3) 2% carrageenan + 1% carbopol + 2% N9, 4) 3% carrageenan + 2% N9, 5) Gynol II® and 6) K-Y PLUS®. Formulations were placed in the vagina of progestin treated mice 10 min prior to inoculation with HSV-2. Infection was determined by the presence of inflammation and lesions in the genital region. Formulations of carrageenan significantly protected mice from infection following vaginal inoculation with HSV-2. OTC spermicides tested were somewhat less effective. Formulations of carrageenan + N9 were significantly more effective than OTC spermicides containing the same amount of N9. Carrageenan formulations with 2% N9 were as effective as OTC products in killing spermatozoa as accessed by the Sander Cramer test. Formulations of carrageenan may be effective in blocking infection by HSV-2 without affecting fertility. Carrageenan based N9 spermicides may be more efficacious than existing spermicides in preventing infection by herpes simplex virus and possibly other enveloped viruses.

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MEDICAL CURE OF RECIDIVING HERPES SIMPLEX VIRUS INFECTION BY MEANS OF PROTEOLYSIS INHIBITORS

A.S.Fedtchouk, P.G.Veveritsa, V.P.Lozitsky, Yu.I.Girlya. *Research Institute of Virology & Epidemiology, Odessa, Ukraine.*

As it is well known the herpes virus is an AIDS-associated viral infection. Besides its own dangerousness the herpes recidiving virus is the model infectious agent which can be used also as the target for new medicine use probing. The stable interest to the simplex herpes virus is caused by the variety of its clinical manifestations, its chronic course and also by the lack of effective medical preparations. That is why the search of new antiviral agents is one of the main tasks of health defense activities. It is known that the proteins of some viruses, including the herpes one, are passing the stage of proteolytic activation during the reproduction stage. Thus, the proteolysis inhibitors application for the herpetic infection cure is generally approved. As it has came out from our laboratory practice, the proteolysis inhibitors use such as E-aminocaproic acid (E-ACA) or para-aminomethylbenzoic acid (PAMBA) in the therapy process of recidiving herpes simplex virus infection allowed to decrease the recidives frequency and shortened the time of its resolution by three or more days. The remissions have become longer more than 2,5 times. The entire patients have demonstrated the immunodeficit in the various stages concerning the T-lymphocytes disfunction. The therapy proposed as a result of our research caused the normalizing of all the immunological indexes. The clinical results obtained during the present work make it possible to recommend the proteolysis inhibitors E-ACA and PAMBA as the medicine for the recidiving herpes cure.

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New Ferrocene Derivatives with Anti-Influenzavirus A Activity

U. Schnittler[1], R. Wyrwa[2], H.-M. Dahse[2], R. Thiericke[2], S. Grabley[2], A. Stelzner[3], M. Schmidtke[3]

[1]University Hospital, Dept. of Cardiology of the Friedrich Schiller University, Erlanger Allee 101, 07743 Jena, Germany, e-mail: Schnittler @POLKIM.MED.Uni-Jena.de, [2]Hans Knöll Institute for Natural Product Research, Beutenbergstr. 11, 07745 Jena, Germany, [3]Institute of Virology at the Clinical Center of the Friedrich Schiller University, Winzerlaer Str. 10, 07745 Jena, Germany

In order to find new antiviral compounds, a number of structurally related ferrocene derivatives was synthesised. Then, 35 selected compounds were tested for antiviral activity against influenzavirus A (H3N2), coxsackievirus B3 (CVB3), and herpes simplex virus type 1 (HSV1). At first, the cytotoxicity of these compounds for HeLa-, MDCK-, and GMK-cells as well as their influence on cell proliferation in L-929 and K-562 cells was tested. For primary antiviral screening, cytopathic effect inhibition assays were applied. The antiviral activity of most active compounds was later confirmed using plaque reduction assays. Guanidin-HCl, foscarnet, amantadine, and ribavirin served as positive controls in each test.

None of the 35 tested ferrocene derivatives inhibited the HSV1- or CVB3-induced cytopathic effect. But three derivatives showed specific inhibition of influenzavirus A replication. Therapeutic indices (ratio of 50 % cytotoxic concentrations versus 50 % inhibitory concentrations) for these three derivatives ranged between 5 and 30. To elucidate possible mechanisms of antiviral action, the effect on cell free virus as well as the influence of the ferrocene derivatives on adsorption and penetration of influenzavirus A were investigated by modified plaque reduction assays. Furthermore, the time-dependent influence of these compounds on the synthesis of viral minus strand RNA as well as haemagglutinine and nucleoprotein mRNA was examined by RT-PCR. Determining the intensities of the respective bands of PCR products after gel electrophoresis, an inhibition of viral RNA synthesis was not detected. Our results demonstrated that all three antiviral active ferrocenes were targeted on cell free virus and inhibited additionally virus binding to host cell membranes. Due to their polar structures, these ferrocene derivatives may be incorporated into the viral membrane, which could decrease the stability of the virus or inhibit virus adsorption.

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The Use Of A Fluorescent Quantitative Dye Uptake Assay As An Anti-Respiratory Syncytial Virus (RSV) Assay. D.L. Barnard, J.E. Matheson, J.H. Huffman and R.W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, UT, USA.

There is a need for a more quantitative assay in which RSV replication can be detected accurately and early. Antiviral assays for detecting inhibitors of RSV can take up to 14 days by visual observation of cytopathic effects (CPE reduction or syncytial reduction [SR] assays), especially if clinical isolates are used. This time can be reduced to as early as 1-2 days post infection using a higher multiplicity of infection. However, the virus concentration may then overwhelm any antiviral effects of the compound. In addition, the CPE assay is not considered as quantitative as other assays and the SR assay often suffers from counting errors, especially as the syncytia get large or if they are very small, pinpoint size. Therefore, experiments were done to evaluate the suitability of commercially available fluorescent dyes for quantifying anti-RSV effects and cytotoxicity of ribavirin in a 96-well format using a fluorescent plate reader. Of the dyes tested to date, SYTO® 11 and 12 (Molecular Probes, Inc.; Eugene, OR, USA) nucleic acid binding dyes were the most effective in detecting early virus infection. Fifty percent effective doses (EC_{50} = 1-4 µg/ml) and 50% inhibitory doses (IC_{50} = 900-1000 µg/ml) derived from these assays were comparable to those calculated from the CPE reduction assay (EC_{50}= 2 µg/ml, IC_{50} = 480 µg/ml) when at least 50% of the monolayer showed virus CPE (2-3 days). These results suggest that SYTO® 11 and 12 dyes may find utility in a fluorimetric antiviral assay that will detect RSV early in the infection and quantify compound cytotoxicity on the same plate.

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Antiviral Activity of New 6-Oxiranyl-, 6-Methyloxiranyluracils, and 4(3H)-Pyrimidinone Derivatives.

A.T. Palamara*, ,P. Savini§, C.Buè§, S. Marini§, R. Saladino°, M.C. Danti°, M. Botta and Enrico Garaci§.
*Dept of Cellular and Molecular Biology and Patology, University of Naples "Federico II", Italy;
§Dept. of Exp. Med. and Biochem. Sc. Univ. "Tor Vergata" Rome, Italy;
° Dept. of Agrochem. and Agrobiol. Univ."La Tuscia" Viterbo, Italy.

During the last few years, there has been a growing interest in the biological activity of substituted pyrimidines and some uracil and pyrimidinone derivatives, substituted either at C-5 and C-6 position, have emerged in the field of antiviral chemotherapy. We have recently reported the synthesis of 5,6-disubstituted-5,6-dihydrouracil derivatives characterized by selective inhibitory activity against Sendai virus (SV). SV is a member of parainfluenza viruses (enveloped viruses with nonsegmented negative-strand RNA genome) that are second only to respiratory syncytial viruses as a cause of serious respiratory tract diseases in infants and children. In the present study, we evaluated the potential antiviral activity of several new synthesized 6-oxiranyl and 6-methyloxiranyluracil and pyrimidinone derivatives on SV infected Madin Darby canine kidney cells. We also tested the cytotoxicity of these compounds in both a monolayer (MDCK), and suspension cell lines (mouse myeloma cells NSO), as well as in primary cell coltures such as normal human lymphocytes. The measure of the haemagglutinating units (HAU) in the supernatant of the infected cells showed that most of compounds analyzed showed interesting inhibitory activity on SV replication with ED_{50} lower than micromolar. Some of the compounds tested showed an associated toxic effect calculated as CC_{50} in proliferating cells, even though such an effect was not microscopically detectable on confluent cells, at the concentration range in which compounds have been found active. On the basis of the obtained results the following structure-activity relationships can be tentatively reported: i) the N,N-dimethyluracil scaffold, very unusual for antiviral compounds, along with the C-6 substitution ion uracil ring, seems to be an important feature for active compounds. ii) The position, the substitution pattern, and the stereochemistry of the oxirane ring play an important role in modulating both the activity and the toxic effect of the products.

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THE THERAPEUTIC AND PROPHYLACTIC ACTION OF PROTEOLYTIC INHIBITOR PARA-AMINOMETHYLBENZOIC ACID DURING EXPERIMENTAL INFLUENZA AND HERPES

V.P.Lozitsky[1], L.E.Puzis[1], A.G.Kolomiets[2], N.D.Kolomiets[2]
[1]Mechnikov Research Institute of Virology and Epidemiology, Odessa, Ukraine; [2]Institute of Epidemiology and Microbiology, Minsk, Belarus

Para-aminomethylbenzoic acid (Ambenum - Am) is the inhibitor of proteolysis especially fibrinolysis. This medicine showed wide spectrum of antiviral activity in vitro. Therefore we studied its activity during experimental influenza and herpes. The mice were intranasally infected with influenza virus A/PR/8/34 (H1N1) in doses that caused the death 70-80% of them. Treatments were begininng 4 hour previrus exposure. Mortality of treated animals was on 20% and 24% lower respectively when we used 1% solution of Am intranasally (0,05 ml once daily during 5 days) or subcutaneously (0,2 ml twice daily during 5 days). Prophylactically we applicated 1% solution Am subcutaneously as describe above and 14 days after beginning treatment mice were infected with the same strain at dilutions ranging from 10^{-1} to 10^{-6} using 4 animals per each dilution. The difference in LD_{50} of control and experimental groups were 2,5 lg (if mice were infected 6 days after using Am) and 1,25 lg (when mice were infected 2 days after injection). Injections of 1% Am (0,3 ml once daily during 10 days) lower mortality of treated animals on 29% or 49% if we infected mice with HSV-1 intranasally or intraperitoneally respectively. Am showed high effectiveness also for treatment herpetic experimental keratokonjunctivitis in rabbits. So Am show not only therapeutic but prophylactic effectiveness during experimental influenza and demonstrate antiherpetic action in some form experimental herpes.

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Effect of Vitamin E Supplementation on the Processes of Lipid Peroxidation in Influenza Virus Infected Mice

M. Mileva[1], L. Tantcheva[2], V. Savov[3], A. S. Galabov[4] and S. Ribarov[1]
Medical University - Sofia[1], Institute of Physiology[2] - Bulg. Acad. Sci., University of Sofia "St. Kliment Ohridski"[3], Institute of Microbiology[4] - Bulg. Acad. Sci., Sofia, Bulgaria

The effect of vit. E supplementation on the processes of lipid peroxidation (LPO) in mice infected with influenza virus A/Aichi/2/68 (H3N2) was investigated. The LPO primary products (conjugated dienes) and the secondary one (malone dialdehyde - MDA) in target tissues on the 5th and 7th day after virus inoculation were measured. The treatement was carried out by i.p. injection of α-tocopherylacetate in daily doses of 60 mg/kg, 120 mg/kg and 240 mg/kg for 5 days before inoculation. It was found that the virus infection increased the level of conjugated dienes 3 times in blood serum and approximately 2 times in the lung and in the liver. The endogenous vit. E content significantly decreased in the infected mice. Influenza virus infection promoted the MDA level about 250% in blood serum, in liver - 200% and in lung - 150%. Our experiments demonstrated that primary and secondary products of LPO decreased when the dose of vit. E increased. Vitamin E supplementation had a regenerating effect on the changes of the parameters: dose of 240 mg/kg inhibited conjugated dienes in blood serum (by 54%), in lung (by 59% on the 5th day and 107% on the 7th day), MDA in blood serum (by 114%). It may be supposed that vit. E reduced the oxidative stress following the experimental virus infection.

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Comparative Efficacy Long and Short Administration of Aerosol Ribavirin Agains RSV Infection in Infants.

Kubar O.I., Gukova E.A., Asatrjan A.G.,
Bryanceva E.A.,
Pasteur Institute, Saint-Petersburg, Russia.

The purpose of this study has been to compare the efficcacy and the safety of the different schemes of aerosol - Ribavirin's administration used in the treatment of RSV infection in infants. In our study two schemes of Ribavirin has been used: 1-st- 12-18 h. daily for 3 days; 2-nd-2 h twice a day for 3 days. Ribavirin (Virazol -ICN, USA) has been administrated by small-particle aerosol. In both groups of infants hospitalized with RSV infections and treated by aerosol Ribavirin, duration of symptoms of disease were the same and shorter compare with the group of the infants with RSV infection which has not been taken by aerosol Ribavirine. The dynamics of laboratory disorders were much better in Ribavirin's groups than those in the contral one. According these results, the short sheme of Ribavirin administration against RSV infections in infants may be recomended for treatment too.

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THERAPEUTIC AND PROPHYLACTIC EFFECTIVENESS OF UNITHIOLUM DURING EXPERIMENTAL INFLUENZA

A.Yu.Sudakov, T.L.Gridina and Yu.I.Girlya
Mechnikov Research Institute of Virology and Epidemiology, Odessa, Ukraine

Disulphide bonds have a extreme significance in the virus-cell innteractions. They are stabilising elements as for proteins incorporated in the viral surface and as well for receptor proteins of plasmatic membranes of sensitive cells. It seems quite logical to suppose that the disulphide bonds cracking has the antiviral effect. We have discovered the antiviral activity of Unithiolum officinal preparation (in a medical practice it is usually used as an antidote when poisoning by thiols poisons takes place). This preparation hinders the influenza A and B viruses reproduction in the tissue culture. The intranasal therapeutical or prophylactic using of Unithiolum (50 mg/ml) effectively protects the mices from lethal cases during the experimental influenza. We have investigated some of mechanisms of antiinfluenza action of Unithiolum. We have discovered that Unithiolum decreases the proteolytic activity in preparations of purified and concentrated influenza virus and also in preparation of cell membranes of chorioallantoic membranes of chick embryos.

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EFFECT OF α-TOCOPHEROL ON THE PROCESSES OF FREE RADICAL LIPID PEROXIDATION AND LIVER MONOOXYGENASE ACTIVITY IN EXPERIMENTAL INFLUENZA VIRUS INFECTION

L. Tantcheva[1], M. Mileva[2], V. Savov[3], A. S. Galabov[4], A. Braykova[5]

Institute of Physiology[1], Institute of Microbiology[4], Institute of Molecular Biology[5] - Bulgarian Academy of Scienses, Medical University - Sofia[2], University of Sofia "St. Kl. Ohridski"[3], Sofia, Bulgaria.

Acute virus infections are accompanied with large changes in cell metabolism, followed by activation of the processes of lipid peroxidation (LPO). White male mice, experimentally infected with influenza virus Aichi/2/68/(H3N2) received the natural antioxidant alpha-tocopherol (vit E) during 5 days in doses 60, 120 and 240 mg/kg b.w. intraperitoneally. Influenza virus infection increased the level of LPO products in the liver: the conjugated diens (2-fold) and the concentration of the free and bound malone dialdehyde (MDA). Hepatic drug metabolism was inhibited - cyt. P-450 content (by 50%), NADPH-cytohrome C-reductase activity (by 53% on the 5th day and by 70% on the 7th day on the virus innoculation), microsomal monooxygenase activity (anilin hydroxylase, ethylmorphine N-demethylase and amidopyrin N-demethylase) (by 33-40%). The correlation between increased LPO products and inhibited drug metabolism was evaluated: cyt P-450/LPO (r=-0.94), NADPH cyt. C-red/LPO (r=-0.67). Vit E pretreatment had a regenerating effect on the changed parameters: the increased LPO products and inhibited metabolism by virus infection were normalized. The effect of doses 120 and 240 mg/kg vit E was the strongest. The mechanism of protective effect of vit E was discussed.

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Fractal Approach to the Evaluation of Sensitive Cells Interaction with the Viral Particles.

A.P.Fedtchouk, A.S.Fedchouk, A.Yu.Sudakov. *Institute of Virology & Epidemiology, Odessa State University, Physical Department, Odessa, Ukraine.*

The promotion of new physical techniques application to the viral infectious processes dynamics evaluation. We have proposed the original method of low-power laser beam diffraction on the thin layers of white mice lungs under the experimental influenza A0\32 infection. We have compared the fractal dimension of the samples of animal lungs cured and non-cured with unithiolum. The viral particles as well as their nearest neighbourhood play the role of the optical inhomogeinity and, in such a way, the centres of laser beam diffraction. We have also controlled the infectious activity of the lungs with the use of the standard methods of titration. As well we have determined the enzymatic activity of the proteolytic systems of lungs preparations. The fractal dimension of the diffraction pattern is being proportional, as it was shown experimentally, to the titration grade of the certain stage of the infectious process. It was discovered that the linear regression interrelation exists between the fractal dimension of the diffraction pattern and the titration grade of the viral infectious process at the any possible given moment of the early stage of virus-cells interaction. Using the proposed method we have shown that the unithiolum plays the role of active protector of the sensitive cells at the early stage of the viral infectious process. By means of the direct optical experiment we have shown that the method of the fractal analysis of the diffraction patterns can be of great use in the evaluation of the new medicine influence on the rate of the infectious processes dynamics without any strong dependence on the real nature of the virus itself and is applicable to the testing process of different new medical preparations.

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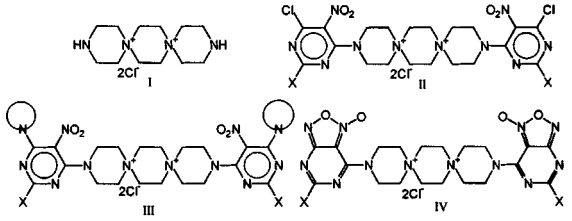
Synthesis of Symm-Heteryl Derivatives of Dispirotripiperazine as Antiviral Agents

V.A. Makarov#, M. Schmidtke*, V.G. Granik#
State Scientific Centre «NIOPIK», Moscow, 103787, Russia
* Institut fur Virologie, Klinikum der FSU, Jena, D-07745, BRD

Symm-heteryl derivatives of 3,12-diaza-6,9-diazonia(5,2,5,2)dispiro-hexadecane by the common structure II was synthesized by the reaction of nucleophilic substitution of dispirotripiperazine I with 2-R-4,6-dichloro-5-nitropyrimidines in alkaly conditions. Further modification of this system through substitution of 4-chlor atom in pyrimidine ring got several compounds III and IV. The 50 % cytotoxic concentrations determined spectrophotometrically on confluent HeLa-, MDCK-, and GMK cell monolayers ranged from >100 μM to >1 mM. Values for the 50 % inhibitory concentration againstcoxsackievirus B3 (CVB3), influenzavirus A and herpes simplex virus type 1 (HSV1) have been calculated for each compound afterspectrophotmetrical analysis of protective activity. Some Symm-Heteryl derivatives of Dispirotripiperazine II-IV inhibited moderately the CVB3-induced CPE. Surprisingly, the investigated compounds were potent inhibitors of HSV1 replication at concentrations less than 10 μM. Therefore, they may be potential candidates for the development of antiherpetic agents.



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ADAMANTANE CONTAINING ANTIVIRALS: COMPARATIVE STUDY OF THE KNOWN DRUGS AND NEWLY DEVELOPED POLYANIONIC DERIVATIVES.

A.V. Serbin[1], L.L. Stotskaya[2], Klimochkin Yu.N.[1], I.F.Barinsky[3], A.G. Bukrinskaya[3], K.N. Kozeletskaya[4].
[1]*Biomodulators & Drug Res.Dep. of Health Research & Development Foundation;* [2]*Topchiev Institute of Petrochemical Synthesis;* [3]*Ivanovsky Virology Institute, Moscow, Russia.* [4]*Institute of Influenza & other viral diseases, St.-Petersburg, Russia*

Objectives. To investigate amplification of hydrophobic-targeted anti-viral potential of a polycyclic hydrocarbons as a result of cooperation with multi-point hydrophilic-sensitive activity of the special polyanionic carriers. To study resourses of the adamantane derived drugs redesign.

Methods. The substances (**AMANTs**) were synthesized by conjugation of adamantane (**Ad**) derivatives with maleic acid copolymers (**Pc**, MW<15KD) to membranotropic structures of type **Ad-Spaser-Pc**. **AMANTs** were evaluated as candidates to new generation of antivirals and compared with traditional adamantane containing drugs amantadine (**Am**) and rimantadine (**Rim**).

Results. As **Am** and **Rim**, the optimal designed representatives of **AMANTs** manifest anti-influenza A activity. However, unlike **Am** or **Rim**, the new antiviral agents possess much lower toxicity on molecular, cellular, tissues and animals organism levels without by effects on electric balance of epithelial (or related) protective bio-barrieres and undesirable affinity to CNS. Furthermore, the **AMANTs** exhibit new valuable antiviral activities, which are not typical for the known **Ad**-derived drugs: inhibition of **Rim** (**Am**)-resistant ***B***-type ***influenza*** virus; blocking of ***HIV-1*** strains replication on lymphoblastoid cells and macrophages; preventive protection of animals against ***eastern equine encephalomyelitis***, ***tick-borne encephalitis*** and ***rabies*** viruses. The letter was achieved by pretreatment of mice with **AMANT** and especially by using of it as an adjuvant of vaccines.

Conclusions. Redesign of adamantane drugs to new molecular structures based on polyanionic matrixes allows to regulate specificity of interaction with biomolecular targets results in lower toxicity and amplified antiviral potential. Particularly, the *combination* of anti-**HIV** with anti-(*influenza and other viruses*) activity could be vital for prophylaxy and therapy of **AIDS**.

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Oral Activity of 1-O-Hexadecyl-propanediol-3-phospho-acyclovir (HDP-ACV) and HDP-P Ganciclovir (HDP-GCV) in Herpes Simplex Virus (HSV) and Murine Cytomegalovirus (MCMV) Infections of Mice.

R. J. Rybak[1], K.Y. Hostetler[2], J.R. Beadle[2], C. Hartline[1], and E.R. Kern[1]. [1]University of Alabama School of Medicine, Birmingham, Alabama, and [2]University of California, VA Medical Center, San Diego, California, USA.

Herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infections can cause a wide range of clinical manifestations. While ACV and GCV are therapeutically effective, oral bioavailabilities are low, thus, large doses are necessary and GCV requires i.v. administration. The alkyl ether phospholipid analogue of ACV, HDP-ACV, was recently found to have 100% oral absorption in mice. This observation led to the synthesis of the corresponding GCV analogue, HDP-GCV. The purpose of these studies was to evaluate the efficacy of these new compounds against HSV and CMV in tissue culture and in HSV-1 and MCMV infections in mice. *In vitro*, HDP-ACV was five- ten fold less active than ACV whereas HDP-GCV had equivalent activity to GCV against HSV-1 using a DNA reduction or plaque reduction assay. Against HCMV, HDP-GCV was more active than GCV, however, it was considerably less active than GCV against MCMV. *In vivo*, HDP-ACV and HDP-GCV were administered orally twice daily to BALB/c mice infected intranasally with HSV-1. Significant protection versus placebo was demonstrated with doses of HDP-ACV at 100, 50, and 25 mg/kg and was similar to ACV. HDP-GCV was highly effective against HSV-1 in doses ranging from 25 mg/kg to 1.28 mg/kg, which was also similar to GCV. In addition, HDP-GCV was evaluated against MCMV in mice and exhibited significant activity at concentrations ranging from 30 mg/kg administered once or twice daily to 10 mg/kg twice daily. This was generally comparable to GCV. These data indicate that HDP-GCV has activity similar to GCV in mice and should be further evaluated to assess its potential for use in humans.

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A THIOSEMICARBAZONE DERIVATIVE INHIBITS CMV REPLICATION AND EBV TRANSFORMATION OF CORD BLOOD LYMPHOCYTES

M Margalith[1], M. Simkin[1], T. Eini[1], S. Berstein[1], and Y. Teitz[2]
Dept. of Virology[1] Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.
Dept. of Human Microbiology[2], Sackler School of Medicine, Tel-Aviv, Israel.

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are common human pathogens belonging to the Herpesviridae. In the immunocompromised ,including organ transplant patients, cancer patients and AIDS patients, CMV may cause severe localized or disseminated life threatening diseases, and EBV may induce lymphoma development. The currently available anti-CMV drugs: ganciclovir, foscarnet and cidofovir, which interfere with viral and cellular DNA synthesis, have toxic side effects, and clinical resistance emerges. There are no anti-EBV drugs available. In the search for alternative drugs to inhibit CMV at a different stage of viral replication we have demonstrated that a thiosemicarbazone derivative A-IBDAT (8μg/ml) inhibits CMV (Towne strain) replication in 93% of infected human foreskin fibroblasts as determined by CMV antigens and infectious virus. A-IBDAT (2μg/ml) inhibits formation of EBV transformed cord blood lymphocytes colonies by 59%. In both virus cell systems a dose related inhibition by A-IBDAT was detected.

Thiosemicarbazone derivatives were chosen since methyl Isatin β thiosemicarbazone (m-IBT) inhibited poxviruses and M-IBDET inhibited retroviruses specifically at the level of viral protein synthesis. In cord blood lymphocytes there was no inhibition of cellular actin synthesis by A-IBDAT at concentrations that inhibit EBV transformation. A-IBDAT in human osteosarcoma cells containing integrated HIV-1 defective genomes had no effect on HIV-1 P-24 production at concentrations inhibiting CMV replication in these cells. Further studies are needed in order to establish A-IBDAT as a drug for treatment.