Oral Session I: Retrovirus Infections I

1

Synthesis and Potent Anti-HIV Activity of Novel 2'-Fluoro-4'-Thio-Nucleosides

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Although a number of antiviral nucleosides are currently available for the treatment of HIV infection, their toxicity and drug resistance as well as patient compliance problems due to the inconvenient dosage forms, warrant further drug discovery and development of new nucleosides as anti-HIV agents. As part of our continuing efforts to discover anti-HIV agents to overcome these issues, we have recently initiated the synthesis and biological evaluation of 4'-thio-nucleosides. Three types of 4'-thiocytosine nucleosides were synthesized; 2',3'-dideoxy-2'-fluoro-4'-thio D- & L-cytosine nucleosides and their corresponding unsaturated nucleosides. For the synthesis of 2',3'-dideoxynucleosides, 2-fluoro-2,3-unsaturated γ-lactone was used as the starting material. The lactone was stereoselctively reduced to a saturated lactone, which was converted to 4-thio-lactol in three steps with the 2-fluoro group in the ribo-configuration. Acetylation, followed by condensation of the 4-thio-lactol with cytosine and the standard nucleoside conditions gave the enantiomerically pure L-isomer. For the 2',3'-unsaturated 4'-thionucleosides, we utilized the same lactol used for the 2',3'-saturated nucleoside. The lactol was converted to a lactone, selenylation, reduction and acetylation of the lactone followed by the condensation of the resulting acetate and the standard chromatographic separation and purification afforded the desired 2',3'-unsaturated 2'-fluoro-4'-thio-cytidine. The synthesized 4'-thionucleosides were evaluated for anti-HIV activity and toxicity *in vitro* in human lymphocites. The saturated L-2'-fluoro-4'-cytidine showed only marginal anti-HIV activity. However, both unsaturated D- & L-isomers showed potent anti-HIV activity (0.53 and 0.47 μM, respectively) in human PBM cells with no significant toxicity up to 100 μM. Detailed chemistry and antiviral activity of the syntheiszed nucleosides will be discussed (Supported by NIH-AI32351 and the VA).

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Discovery of Novel Inhibitors of HIV Integrase: Synthesis and Exonuclease Activity

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The retroviral enzyme, HIV-1 integrase, incorporates double-helical HIV DNA into host chromosomal DNA. This enzyme first catalyzes the excision of the two terminal nucleotides at the 3′-end of each strand of viral DNA (endonuclease activity, 3′-processing) leaving recessed ends that terminate with xxCA-OH. In the next step (strand transfer, integration), nucleophilic attack of the 3′-OH on a specific phosphodiester bond results in cleavage of host DNA and subsequent integration of the tailored HIV DNA into host DNA. Some small oligonucleotides of natural origin are capable of interfering with both steps of integration by competing with the viral DNA for binding to HIV integrase. However, small oligonucleotides are rapidly cleaved by cellular nuclease activity. Increasing nuclease stability by chemical alteration of the internucleotide phosphate bond results in decreased integrase activity. We have designed and synthesized non-natural dinucleotides, with specifically distorted internucleotide phosphate bonds, that exhibit nuclease stability and anti-HIV integrase activity. This paper will describe the design, synthesis and molecular modeling studies on these compounds. Enzymology with 3′- and 5′-exonucleases will be discussed and nuclease-resistance data will be explained. Anti-HIV-1 integrase activity will be presented and discussed in terms of recognition and potency of inhibition.

3

Synthesis and Activity of a Novel Series of HIV Protease Inhibitors Containing Inverted Stereochemistry at P_1.

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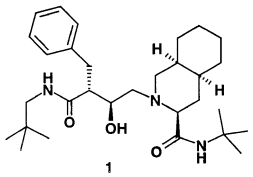
As part of our efforts to identify low molecular weight inhibitors of HIV protease we have discovered a novel class of inhibitor represented by compound 1. This compound with an IC_{50} in an isolated enzyme assay of 20nm contains the unusual feature of inverted stereochemistry at P_1 compared to known inhibitors and substrates. Compounds of this class are readily prepared from (S)-dimethyl malate and X-ray co-crystal structures revealed an unusual binding mode involving an intramolecular hydrogen bond between the amide carbonyl and the decahydroisoquinoline nitrogen. The synthesis and structure activity relationships of this class of compound will be described.



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4

Structure-Activity Relationships of SJ-3366 (2, 4 (1H, 3H)-Pyrimidinediones): Inhibition of Reverse Transcriptase, Virus Attachment and HIV-1 and HIV-2 Replication.

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We have recently described an N-1 substituted pyrimidinedione, SJ-3366 that inhibits both HIV-1 RT and virus entry. We undertook to specifically examine the structure activity relationships of SJ-3366 for inhibition of HIV-1 and HIV-2 replication as well as virus entry and anti-HIV-1 RT activity by homocyclic substitutions at N-1 of the pyrimidinedione. Seventy three cyclic N-1 substitutions were made, including cyclo-propyl, -butyl, -pentyl and 1, 2 or 3 –cyclopenten–1-yl. The analogs were evaluated for anti-HIV-1 and HIV-2 antiviral activity in a cytoprotection assay with CEM-SS cells. Analogs were also assessed for inhibition of HIV RT in a biochemical assay and virus attachment in HeLa CD4 LTR β-galactosidase cells. In general separation of the cyclic moiety from the pyrimidinedione backbone by an ethyl versus a methyl group resulted in less antiviral activity. In analogs employing the methyl spacer, substantially improved 50% inhibitory concentrations over SJ-3366 were identified (IC50 or I50) for HIV-1 (20-fold lower), HIV-2 (160 –fold lower), RT (60-fold lower) and attachment (10-fold lower) inhibition. Several analogs were found to be non-toxic at a high test concentration of 1.1 mM yielding therapeutic indexes (>500,000) equal to or better than SJ-3366. Finally, several of these analogs were assessed against fully resistant SJ-3366 resistant virus and found to retain antiviral activity. Thus, N-1 substituted pyrimidinediones are highly potent and unique inhibitors of HIV-1 and HIV-2 replication.

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Mechanistic Studies of Dioxolane Guanosine 5'-Triphosphate: Implications for Efficacy, Lack of Cross-Resistance and Selectivity of DAPD.

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DAPD, a prodrug of dioxolane guanosine (DXG), is a nucleoside analog currently in Phase I/II clinical trials for the treatment of HIV infection. DAPD is deaminated by adenosine deaminase to DXG, which is subsequently phosphorylated to the corresponding 5'-triphosphate, DXG-TP. Steady-state and pre-steady-state kinetics were used to evaluate the activity of DXG-TP against wild-type and resistant HIV-1 RT and human cellular DNA polymerase α, β and γ. DXG-TP was shown to be a potent alternative substrate inhibitor of the HIV RT with a K_i value of 0.019 ± 0.002 μM, whereas the K_i values for DXG-TP with the human DNA polymerases α, β and γ were 78 ± 17, 32 ± 2, and 4.3 ± 0.4 μM, respectively. Pre-steady-state kinetic studies demonstrated that the binding affinity of DXG-TP for the RT was similar to that determined for dGTP with either a DNA or RNA template. However, dGTP was incorporated 11 and 23 times faster than DXG-TP when the template was DNA or RNA, respectively. Comparing the overall efficiency of incorporation of dGTP with that of DXG-TP showed that the RT incorporated DXG-TP only 17 times less efficiently than dGTP. Furthermore, the overall efficiency of incorporation of DXG-TP was not affected by mutations conferring resistance to zidovudine or lamivudine. To give further insight into the origin of the selective activity of DAPD/DXG, steady-state kinetic studies with human DNA polymerases α, β and γ were performed. DXG-TP was a relatively weak inhibitor of human DNA polymerase α and β, however DNA polymerase γ proved to be somewhat sensitive to inhibition by DXG-TP. Nevertheless, mitochondrial toxicity studies performed using HepG 2 cells showed that DXG was not toxic to mitochondria at concentrations up to and including 50 μM. A pre-steady-state kinetic study showed that DXG-TP was a poor substrate for DNA polymerase γ compared with dideoxyCTP, a potent inhibitor of mitochondrial DNA synthesis. Excision of the terminally incorporated DXG-MP by the γ polymerase-associated exonuclease was not observed during a 12-hour incubation. These data provide a mechanistic explanation for the overall activity and lack of preclinical toxicity for DAPD/DXG and the lack of *in vitro* cross-resistance to viruses resistant to lamivudine and zidovudine.

6

Identification of a Small Molecular HIV-1 Fusion Inhibitor Targeted to the gp41 N-terminal Heptad Repeat Region

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HIV-1 envelope glycoprotein gp41 play a critical role in the fusion of viral and target cell membranes. The gp41 C-terminal heptad repeat (CHR) region interacts with the N-terminal heptad repeat (NHR) region to form a six-stranded fusion active gp41 core structure. Previously, we found that peptides derived from gp41 CHR region, designated C-peptides, are potent HIV-1 fusion inhibitors (Nature 365:113, 1993) by interacting with gp41 NHR region. They have been developed as novel anti-HIV-1 drugs. However, C-peptides cannot be orally administered and are extremely expensive. It essential to develop small molecular HIV-1 fusion inhibitors with a similar mechanism of C-peptides. Recently, we have identified a small molecular organic compound, designed ADS-J1, which inhibits HIV-1-mediated membrane fusion by targeting the gp41 NHR region, like the C-peptides. This active compound contains both hydrophobic and acidic groups while the inactive compounds only have hydrophobic groups. Analysis by computer modeling indicate that the acidic groups in the active compounds can form salt bridge with Lys 574 in the N-terminal coiled-coil region of gp41. Asp 632 in a C-peptide can also form a salt bridge with Lys 574. Replacement of Asp 632 with positively charged residues or hydrophobic residues resulted in significant decrease of HIV-1 inhibitory activity. These results suggest that a salt bridge between an N-terminal coiled coil of the gp41 and an antiviral agent targeted to the gp41 coiled-coil region is important for anti-HIV-1 activity.

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Preliminary Profile of the Antiviral Activity, Safety and Pharmacokinetics of DMP 450, a Novel Cyclic Urea Protease Inhibitor.

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BACKGROUND: DMP 450 is a non-peptidomimetic, water soluble, cyclic urea that is a selective inhibitor of HIV-1 protease. DMP-102 is a phase I/II, randomized, indinavir-controlled, dose escalation study to evaluate the antiviral activity, safety and PK/PD correlation of DMP 450.
METHODS: Antiretroviral naïve patients with HIV RNA >10,000 copies/ml and unrestricted CD4 counts were randomized to receive DMP 450 at three different doses or indinavir 800mg TID, in combinations with stavudine and lamivudine. Fifteen patients (12 DMP 450, 3 indinavir) were enrolled in each cohort at the following doses; Cohort 1 (A) 750mg TID, Cohort 2 (B) 1250mg BID, and Cohort 3 (C) 1250mg TID. Patients receiving indinavir are described as D.
RESULTS: Enrollment has been completed and the on treatment periods range from 24-56 weeks. The median $\log_{10}$ viral load at baseline is A) 4.61, B) 4.99, C) 5.25 and D) 4.93. The percentage of patients at Week 24 , intent to treat, with HIV-1 viral load less than 50 copies/mL are A) 75, B) 80, C) 76.9 and D) 70. Preliminary data indicate that DMP450 is well tolerated; no significant laboratory toxicities have been observed. Most AE's have been mild and self-limited. The study is ongoing and updated data, to include pharmacokinetics, will be presented.
CONCLUSION: To date, DMP 450 appears to have good antiviral activity and tolerability at all doses tested.

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TMC120 (R147681), a Next Generation NNRTI, Has Potent In Vitro Activity Against NNRTI Resistant HIV Variants Including Recombinant Clinical Isolates. M.-P. de Béthune[1], K. Andries[2], D. Ludovici[3], P. Lewi[4], H. Azijn[1], M. de Jonge[4], J. Heeres[4], K. Hertogs[5], M. Kukla[3], P. Janssen[4], R. Pauwels[1]. [1]Tibotec, Mechelen, Belgium; [2]Janssen Res. Fndn., Beerse, Belgium; [3]Janssen Res. Fndn., Spring House, PA; [4]Janssen Res. Fndn.,Vosselaar, Belgium; [5]Virco, Mechelen, Belgium

NNRTIs are potent antiretrovirals but extensive cross-resistance is observed among approved inhibitors of this class. The next generation of NNRTIs should have potent activity against variants resistant to NNRTIs currently in use. TMC120 (R147681), a dianilinopyrimidine derivative, was selected from >300 triazine and pyrimidine analogues of a lead compound in a process that included simultaneous screening against wild type HIV-1 and NNRTI resistant strains with clinically important mutations. TMC120 exhibits IC_{50}s of 0.9nM, 4.1nM and 7.6nM against wild type virus, and the K103N and the Y181C mutants respectively. The potency of TMC 120 is not influenced by α_1-acid glycoprotein (2mg/mL) and moderately affected by 45mg/mL human serum albumin (20-fold increase of IC_{50}). In vitro selection experiments show slower virus emergence than with the current NNRTIs and the need for 2 mutations to observe a significant decrease in the antiviral activity of the compound. When challenged against more than 400 NNRTI resistant recombinant clinical isolates, TMC120 could inhibit more than 50% of these variants with an IC_{50} below 10nM. A role for polymorphisms in highly resistant HIV is suspected. TMC120 has a favourable in vitro activity profile against NNRTI resistant variants. This activity, combined with encouraging safety and pharmacokinetic profiles, has led to Phase II testing of TMC 120.

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The Novel Non-Nucleoside Reverse Transcriptase Inhibitor TMC120 Has Potent Antiretroviral Activity After Short-term Monotherapy in Treatment Naïve, HIV-1 Infected Subjects

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TMC120 (R147681), a dianilinopyrimidine derivative, is a novel, non-nucleoside reverse transcriptase inhibitor (NNRTI) with equipotent *in vitro* activity (IC_{50} = 1-10 nM) against wild-type HIV-1 and NNRTI-resistant variants encoding K103N, Y181C or G190A/S mutations, with only a 20-fold protein binding effect. The antiretroviral activity of TMC120 placebo after short-term monotherapy was assessed in treatment-naïve HIV-1 infected patients (HIV RNA: 5,000-125,000 cps/ml). In a randomized, double-blind Phase I/II study, patients received twice daily oral treatment with placebo or TMC120 (50 or 100mg) as monotherapy for 7 days. Triple therapy with new antiretrovirals was offered thereafter. A total of 43 patients (incl. 9 females) were enrolled, with a median age of 24 years. Median baseline (BL) characteristics were: CD4 cell count: 571 cells/μL; HIV-1 RNA: 40,258 copies/ml. All patients but one (withdrawn because of acute HCV infection) completed the trial. Trough plasma levels (12h after last intake) ranged from 66 to 180 ng/ml after 50 mg BID, and from 64 to 362 ng/ml after 100 mg BID. TMC120 was safe and well tolerated. Reported adverse events (headache, somnolence, insomnia, anxiety) were mild to moderate. Mean changes in HIV-1 RNA (log10 cps/ml) after 7 days, and mean decay rates (log10 cps/ml/day), respectively, were: –0.17 and –0.02 for placebo (n=15); –1.44*** and –0.213*** in the 50mg group (n=13); –1.51*** and –0.237*** in the 100mg group (n=15) (***: $p<0.001$ *vs.* placebo, ITT; no difference between 50mg and 100mg groups). These results demonstrate that TMC120 administered twice daily at doses of 50 and 100 mg for 7 days is a safe and potent antiretroviral agent in treatment-naïve HIV-1 infected subjects.

Oral Session II: Hepadnavirus Infections I

10

A class of imino sugars that prevents hepatitis B virus (HBV) and BVDV replication through possible novel mechanisms - therapeutic implications

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The action of an imino sugar that lacks the ability to inhibit N-linked glycoprocessing but retains activity against HBV and the HCV surrogate, BVDV, production is described. Imino sugars such as N-butyl deoxynojirimycin (N-butyl-DNJ) that inhibit the endoplasmic reticular (ER) glucosidase (which mediate the first steps in N-linked glycoprocessing) have been shown to prevent HBV secretion and cause the intracellular accumulation of viral DNA. N-nonyl- deoxynojirimycin (N-nonyl -DNJ), a nine carbon alkyl derivative of DNJ, is 40 - 100 times more potent at inhibiting HBV secretion than is N-butyl-DNJ. Although N-nonyl-DNJ is an inhibitor of the ER glucosidase, here it is shown that N-nonyl-DNJ retained antiviral activity at concentrations that had no significant impact upon ER glucosidase function. Taken together, these results suggested that N-nonyl-DNJ possessed an antiviral activity attributable to a function other than an impact upon glycoprocessing. This hypothesis was confirmed by experiments showing that an imino sugar that does not alter glycoprocessing, called SP226, retains anti-HBV activity. The possible mechanism and point of antiviral action of N-nonyl-DNJ in the viral life cycle and implications about the relationship between virion intracellular location and replication as well as possible applications as a novel antiviral agent are discussed.

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SeqHepB: A Hepatitis B virus Sequence Analysis Program for Identifying HBV Antiviral-Resistant Mutants in Longitudinal and Cross-Sectional Studies. A Bartholomeusz[1], A Ayres[1], M Littlejohn[1], S Bowden[1], A Edgley[2], P Angus[3], G MacCaughan[4], R de Man[5], M Manns[6], H Tillmann[6], C Trautwein[6], T Bock[6], GKK Lau[7], H Isom[8], SA Locarnini[1] *and Members of the Collaborative Consortium.*

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The SeqHepB system is a Web based program comprising a large hepatitis B virus (HBV) genotype database which has been designed to process data from hepatitis B infected patients for the purpose of providing support to Healthcare professionals. In particular, the system is specifically designed for managing HBV sequencing data for the treatment of these patients with antiviral agents, such as Lamivudine. The analysis and early identification of resistance mutations is of importance especially in the treatment of those patients who experience a virological breakthrough on therapy, and in particular the sub-group of individuals whose HBV DNA levels rise to greater than pretreatment levels. The SeqHepB genotypic resistance analysis utilizes algorithms of drug susceptibility based on the absence or presence of key mutations. The program can also be used to determine the HBV genotype and to analyze other clinically important HBV variants such as those encoding a pre-core mutation, vaccine escape mutation, or deletions in Pre-S by comparison to reference sequences from genotypes A-G. The proprietary web based program allows the easy input of sequence data for the rapid analysis of HBV data in cross-sectional or longitudinal studies, and it is the only program available to date designed specifically for HBV mutational analysis. The SeqHepB system is an important tool for individualized patient management and will be a useful guide to antiviral therapy as new agents and combinations thereof are introduced, and new HBV resistance mutations are identified.

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Post-Clevudine (L-FMAU) suppression of Woodchuck Hepatitis Virus (WHV) covalently closed circular (ccc) and WHV total (t) DNA. S.L. Sacks[1], A. Dicaire[1], M. Singh[1], A. Wen[1], and B. Korba[2] [1]Viridae Clinical Sciences, Inc., Vancouver, BC, Canada and [2]Georgetown University Medical Center, Rockville, MD, USA.

Previous studies in WHV-carrier woodchucks (Korba, et al., *Antivir Res.*41:A54) showed that the antiviral effects of L-FMAU persisted after drug withdrawal in 3/4 animals treated for 12 weeks. To explore mechanisms related to variability of the sustained antiviral effect, levels of WHV cccDNA were measured in serial liver biopsies obtained over 80 weeks. For this, we established a novel assay for WHV cccDNA using the LightCycler™ where single strands of relaxed circular (RC-DNA) are digested with ATP-dependent DNase. Assay sensitivity was determined using cloned, full-length WHV DNA, while specificity of primer pairs was analyzed using WHV-positive serum. A log range of 5.0×10^1 to 1×10^8 was established for both cccDNA and tDNA. Specificity was maintained up to 1:100,000 ratio of ccc:RC-DNA. A synthesized clone was added to the samples to account for tube to tube variation and as inhibitor control. Prior to L-FMAU, WHV cccDNA was detected in 4/4 animals. After 12 weeks initial therapy, cccDNA levels declined to below the limit of detection of our assay and remained at reduced levels in the 3/4 animals that exhibited sustained reductions in WHV replication and gene expression for up to 68 weeks post-treatment. Pretreatment cccDNA levels were highest (78 copies per cell) in the animal that relapsed after therapy compared with 5.9 to 52.8 copies per cell for the 3 non-relapsing animals. This study demonstrates the potential importance of sensitive quantitation of both pre- and post-treatment cccDNA in evaluations of a sustained antiviral response in the treated WHV-carrier woodchuck model.

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Treatment of Chronic Type B Hepatitis with Clevudine Followed by Vaccine Enhances, Broadens Immune Response and Delays Disease Progression in the WHV/Woodchuck Model. BE Korba[1], PJ Cote[1], S Menne[2], IA Toshkov, JL Gerin[1] and BC Tennant[2]. [1]Georgetown Univ. Med. Ctr., Rockville, MD, USA; [2]College of Vet. Med., Cornell Univ., Ithaca, NY, USA

Previous studies in the woodchuck model of HBV infection demonstrated that the nucleoside analogue Clevudine (CLV, L-FMAU) had potent antiviral activity in chronic woodchuck hepatitis virus (WHV)-carrier woodchucks . In 75% of animals treated for 12 weeks, the antiviral effect persisted after drug withdrawal. However, these animals still harbored low levels of replicating virus and do not seroconvert to anti-WHs. We sought to determine whether CLV treatment followed by conventional WHsAg vaccination provided added antiviral benefit through the induction of host immune responses that are normally suppressed during chronic WHV infection. Groups of 8 chronic carrier woodchucks received (1) 10mg/kg/day of CLV orally for 32 weeks followed by WHsAg vaccine (serum-derived, formalin-inactivated, alum) 50 ug/dose, *s.c.* at wks. 32, 36, 40 and 48, (2) CLV for 32 wks. and 4 saline injections, (3) placebo for 32 wks. and 4 doses of vaccine, and (4) placebo and saline injections. CLV treatment induced remarkable ($>10^7$) reductions in viremia and antigenemia (10^2 to $>10^4$), and hepatic WHV antigen expression ($>10^2$) within 12 weeks of drug treatment. Viral markers remained suppressed upon drug withdrawal in 75% of animals in both the vaccinated and non-vaccinated groups. Virus markers were stable in both the control and vaccine only groups. Analysis of host immune response to vaccine demonstrated a broad response at both the cellular and humoral levels in the combined treatment group, which resembled that typical of recovery from acute infection, including the development of anti-WHs antibody that persisted for up to one year. Combined therapy resulted in statistically significant delays in the development of HCC and in the progression of portal hepatitis. This study shows the enhanced benefit of the combination of chemotherapeutic and immunotherapeutic approaches on the progression of chronic hepadnaviral diseases. The woodchuck/WHV model provides an experimental platform for further development of this treatment strategy.

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Synergistic Antiviral L-Nucleosides Specific for Hepatitis B Virus Infection

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β-L-2'-Deoxythymidine (β-L-dT) and β-L-2'-deoxycytidine (β-L-dC) showed potent, selective and specific anti-hepatitis B virus activity *in vitro* and *in vivo.* In combination, these two new investigational agents also showed potent antiviral synergy in combination studies *in vitro* and *in vivo* in the woodchuck model of chronic hepatitis B virus infection. In cell-based assays of HBV replication, a 1:1 molar ratio of L-dT and L-dC was highly synergistic as measured by a reduction in HBV replicative intermediates and extracellular virus production. There was no competition for activation of L-dT and L-dC to their intracellular triphosphate (-TP) forms, which reached levels up to 100 times the EC_{90} of the virus and had a $t_{1/2}$ of 15 hours. *In vivo* pharmacokinetic studies in monkeys have shown that the oral bioavailability of a combination of L-dT and the valine-ester prodrug of L-dC is similar to that of either drug alone. A 12-week study of the combination of L-dT and L-dC was also conducted in the woodchuck model of chronic HBV infection. Plasma viral load was reduced $\geq$8 logs (limit of detection, 300 genome equivalents/ml) with a single oral daily dose of 1 mg/kg/day of L-dT and L-dC in combination. There was also a marked reduction in HBsAg that paralleled the decrease in viral load. There were no safety concerns and end-of-treatment liver biopsies were negative for microvesicular steatosis (fatty change). L-dT and L-dC used alone and in combination are expected to offer new therapeutic options for patients with chronic HBV infection.

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Improved efficacy of 9-(2-phosphonylmethoxyethyl)adenine against hepatitis B virus by carrier-mediated delivery.

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We aim to selectively deliver 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) to parenchymal liver cells, the primary site of hepatitis B virus (HBV) infection. PMEA is effective against HBV *in vitro*, but accumulates *in vivo* in the kidneys, whereas only a small amount is taken up by the liver. To enhance the hepatic uptake of PMEA, we incorporated a lipophilic PMEA prodrug (PMEO-LO) into reconstituted lactosylated high density lipoprotein (LacNeoHDL), a lipidic carrier that is specifically taken up by the asialoglycoprotein receptor on parenchymal cells in the liver. LacNeoHDL-associated PMEA-LO was injected into in rats, and 68.9 ± 7.7% of the injected prodrug was delivered to the liver, whereas < 2% was recovered in kidneys (free PMEA < 5% and > 45%, respectively). The hepatic uptake of PMEA-LO-loaded LacNeoHDL occured by parenchymal cells (88.5 ± 8.2% of the liver uptake). Asialofetuin inhibited the liver uptake by > 75%, indicating involvement of asialoglycoprotein receptors. PMEA-LO contains an acid-labile bond. After internalization and processing of PMEA-LO in the acidic lysosomes, PMEA was released, trans-located to the cytosol, and phosphorylated to its active metabolite. The therapeutic potential of LacNeoHDL-associated PMEA-LO was studied using HepAD38 cells (HBV-transfected HepG2 cells). LacNeoHDL-associated PMEA-LO effectively inhibited HBV DNA synthesis. The EC_{50} value was 35 times lower than that of free PMEA (3.4 ± 0.4 vs. 120.0 ± 17.9 ng/ml). In conclusion, incorpo-ration of PMEO-LO into LacNeoHDL induces high uptake of the prodrug by parenchymal liver cells, whereas the renal uptake is substantially reduced. We found that carrier-associated PMEA-LO effectively inhibits HBV replication, and the targeting of PMEA-LO to the liver is therefore likely to result in an enhanced therapeutic efficacy against HBV, concomittant with a reduced renal toxicity.

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Pharmacokinetics of β-L-2'-deoxycytidine prodrugs in monkeys.

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β-L-2'-deoxycytidine (L-dC) is a potent and specific inhibitor of hepatitis B virus (HBV) replication *in vitro* and *in vivo*. To improve oral bioavailability (~16% in monkey), three ester prodrugs of L-dC (di-O-acetyl, mono-, di-valine) were synthesized and pharmacokinetic parameters determined. The levels of L-dC in plasma and urine after oral dosing of each of the prodrugs was determined in cynomologus monkeys. [^{3}H]-Labeled drug was administered as a single oral dose of 10 mg/kg. Plasma was collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 8 and 24 hr after dosing and drug levels were quantitated using high-pressure liquid chromatography (HPLC) and in-line radioactivity detection. Pharmacokinetic parameters were estimated by model independent methods using the trapezoidal rule. The 3'-mono- and 3',5'-di-L-valine ester derivatives of L-dC produced the highest plasma levels relative to an equivalent dose of L-dC. The C_{max} and AUC for L-dC achieved with orally administered di-val-L-dC were significantly greater (2.5-5 fold) than those estimated for an equivalent dose of L-dC. The plasma half-life of L-dC derived from di-val-L-dC was similar to L-dC (~1.75 h). After oral administration, di-val-L-dC is rapidly and completely converted to L-dC and provides high plasma concentrations of L-dC compared to that following oral administration of L-dC. Intracellularly, the triphosphate form of L-dC reached levels up to 100 times the EC_{90} of HBV with a half-life of 15 hours. The improved oral bioavailability of di-val-L-dC and increased systemic delivery of L-dC make it an ideal candidate for further studies as an anti-HBV agent.

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Comparative Viral Dynamics Analysis of the Clearance Kinetics of HBV from Patients with HBeAg+ and HBeAg- (Precore Mutant) HBV Infection During Antiviral Therapy. M. Tsiang, X. Xiong, H. Yang, C. E. Westland, J. Fry, C. Brosgart, E. J. Heathcote, C. Gibbs. Gilead Sciences, Foster City, CA

Immune clearance of HBV may be followed by re-emergence of immune escape variants with mutations in the precore region or basal core promoter (BCP) that decrease expression of HBeAg. HBeAg has been proposed to promote chronic infection variously by suppressing the immune response or decreasing replication. However, some studies have suggested that HBV variants with precore or BCP mutations may replicate more rapidly and may be associated with a more severe form of liver disease (fulminant hepatitis). A mathematical model was applied to compare the kinetics of viral clearance between patients with HBeAg+ and HBeAg- (precore mutant) HBV infection. Twelve patients with HBeAg+ HBV infection (mean baseline HBV DNA = 4.5 x 10^8 copies/mL; mean baseline ALT = 104 U/L) and eight patients with HBeAg- HBV (8/8 precore, nt 1896, and/or BCP, nt 1762 or 1764, mutations) infection (HBV DNA = 2.1 x 10^8 copies/mL p= 0.33; ALT = 133 U/L p = 0.36) were treated with adefovir dipivoxil 30 mg qd for 12 weeks. Serum HBV DNA was determined at weeks 0, 1, 2, 4, 8, 10 and 12 using a PCR-based assay. Both patient groups experienced a decrease in serum HBV DNA of ~4.0 log10 by week 12. The clearance displayed a characteristic biphasic pattern previously observed in patients treated with varying doses of adefovir dipivoxil, lamivudine or lamivudine + famciclovir combination therapy. The model attributes the first rapid phase to clearance of viral particles whereas the second slower phase reflects the loss of infected cells. The duration and magnitude of the first rapid phase is determined by the antiviral efficacy ε (where ε = 1.00 for complete inhibition of viral production). The parameters for efficacy, the half-life of the virus and the half-life of the infected cells were determined by curve fitting to the HBV DNA data for each patient. The mean parameter values for the HBeAg+ and HBeAg- groups were determined. The results indicate that there was no significant difference between the two patient groups in the efficacy of adefovir dipivoxil (0.98 vs. 0.97; p = 0.67), the half-life of clearance of viral particles (1.4 vs. 1.6 days; p = 0.42) or the half-life of infected cells (13.4 vs. 13.2; days; p = 0.63). Therefore the mechanisms of HBV and infected cell clearance during antiviral therapy were independent of HBeAg and these data are not consistent with the hypothesis that the HBeAg may function as a suppressor of HBV immune response. Moreover, there was no direct correlation between viral particle or infected cell clearance and levels of ALT or HBV DNA at baseline. Additional analyses of the virological rebound following cessation of therapy revealed no difference in the rate constant of the initial rebound, suggesting very similar viral production rate constants.

Poster Session I: Retrovirus Infections I

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N,N-Disubstituted dihydro-alkylamino-benzyl-oxopyrimidines: New, potent and selective anti-HIV agents in the DABO class.

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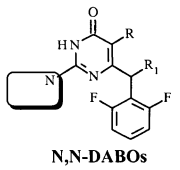
Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) have been reported by our group as non-nucleoside reverse transcriptase inhibitors (NNRTI) in 1992. Since then, huge numbers of oxopyrimidines have been synthesized and tested as anti-HIV-1 agents with the aim to obtain more potent and selective derivatives[1-8]. The structure-activity relationship, supported by molecular modeling, suggested that the presence of C2-alkoxy (O-DABO) or -alkylthio (S-DABO) side chains is a structural determinant necessary for the antiviral activity of these derivatives. Both length and size of the C2 side chain modulate DABO potency. Introduction of a 2,6-difluoro substituent at the C6 phenylmethyl moiety of S-DABOs generates a favorable π-stacking interaction with the Tyr188 side chain and resulted in compounds active in the nanomolar range (difluoro-S-DABOs, F2-S-DABOs). We now present a novel series of DABO derivatives (see below) bearing the highly favorable 2,6-difluorophenylmethyl moiety at C6 position and characterized by the replacement of the alkoxy/alkylthio side chain at C2 with the isoster dialkylamino side chain (amino-DABOs).



N,N-DABOs

R = R_1 =H, Me, Et
= pyrrolidine, (substituted)piperidine, (substituted) piperazine, morpholine, thiomorpholine, tetrahydroazepine, dialkyl

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Synthesis and study of antiviral *S*-acyl-2-thioethyl (SATE) phosphoramidate derivatives of β-L-ddA.

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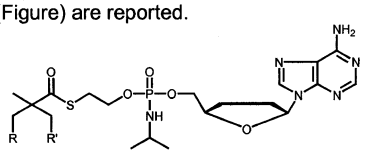
It has been shown that the antiretroviral activity of some nucleoside analogs can be enhanced by the use of their nucleotide prodrugs (pronucleotides). We have previously reported that phosphoramidate derivatives of AZT incorporating one *S*-pivaloyl-2-thioethyl (*t*BuSATE) as biolabile protecting group and an alkylamino group (i.e. isopropyl) allowed the delivery of the corresponding 5'-mononucleotide (AZTMP) into infected cells. Here we apply this approach to β-L-2',3'-dideoxyadenosine (β-L-ddA) through the synthesis and study of its *t*BuSATE phosphoramidate derivative (see Figure). In order to evaluate the potential in antiviral chemotherapy of such phosphoramidates, we studied the effect of SATE chemical modifications on their physicochemical (solubility, log P) and biological (antiviral activity, toxicity, stability) properties. The comparative study of two SATE phosphoramidate derivatives of β-L-ddA which incorporate one or two hydroxylated functions (HOSATE, $(HO)_2$SATE) in the vicinity of the thioester functionality (Figure) are reported.



R = H, R' = H : tBuSATE
R = H, R' = OH : HOSATE
R = OH, R' = OH : $(HO)_2$SATE

The data suggest that further development of this series of SATE phosphoramidates as potential anti-HIV pronucleotide candidates is warranted.

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Antiviral Protides: An Alternative Synthesis of Novel Nucleotide Analogues *via* a Transesterification Reaction

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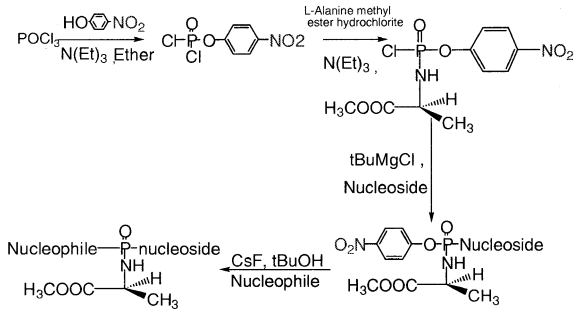
The majority of nucleosides share a common mode of action and must be converted, *via* three successive phosphorylations, to their bioactive 5'-triphosphate forms by cellular or viral induced kinases. The Pro-Tide approach aims to deliver the monophosphate form of the parent nucleoside, in order to by-pass the first phosphorylation step and thus, the need for kinase-mediated activation. Indeed, we have shown that a number of protide analogues have retained full anti-HIV activity in kinase-deficient cells, suggesting that the protides effectively deliver the 5'-monophosphate forms intracellularly. The Pro-Tide concept has been successfully applied to a wide variety of nucleoside analogues, including d4T, d4A and ddA. Our previous methodology which uses established phosphorochloridate chemistry, has some limitations. Thus we sought to find an alternative synthetic route and prepared a number of protide derivatives of potent anti-HIV agents by a fluoride-ion mediated transesterification reaction.



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Novel Synthetic Route towards Cyclic and Acyclic Analogues of AZT Monophosphate (AZTMP)

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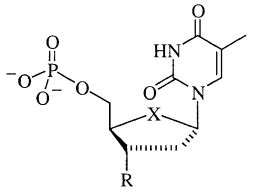
Nucleoside-based inhibitors of reverse transcriptase were the first drugs to be used in the chemotherapy of AIDS. After entering the cell, these substances are activated to their triphosphates by cellular kinases. Thus, for the most extensively used drug, 3'azido-3'deoxythymidine (AZT), whereas phosphorylation is facile, the product is a very poor substrate for the second kinase, thymidylate kinase. Due to the steric demand of the azidogroup in the 3'-position of AZTMP compared to the 3'-hydroxygroup of the natural substrate, 2'-deoxythymidine monophosphate (dTMP), the structure of the enzyme changes, which causes a 200-fold decrease in the rate of phosphorylation.



R = -SCN, -NCS, -F
$-CH_2CH=CH_2$, $-H_2CC\equiv CH_2$
$-CH=C=CH_2$

X = O, CH_2

Here we will present the synthesis of isosters of AZTMP as well as monophosphates of thymine containing analogues of the acyclic nucleoside analogues penciclovir (PCV) and ganciclovir (GCV). The free hydroxygroups of the latter were replaced by groups isoster to the azidogroup in order to investigate the sterical and/or electronical influence of the substituent in this position on the substrate properties towards thymidylate kinase. The monophosphates were synthesized by chemical hydrolysis of the corresponding *cyclo*Sal-pronucleotides, which have been designed to intracellularly deliver monophosphates of anti-virally active nucleosides, on a preparative scale. These phosphotriesters were obtained directly from the corresponding nucleoside analogues as reported previously. The anti-HIV activity of the pronucleotides will be presented.

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Synthesis of Novel Conformationally Restrained Nucleosides

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The conformation of nucleosides is important for optimal binding at a specified enzyme active sites and can therefore have a profound impact on the biological activity. For example: conformational analysis of reverse transcriptase inhibitors has shown that the 3'-exo (and to a lesser extent 2'-endo) character of the sugar moiety with a *trans* (ap) C4'-C5' conformation is the most favourable conformation with regards to biological activity. The introduction of either fused rings (*e.g.* benzofuran) in place of the sugar moiety or the introduction of cyclic moieties (*e.g.* cyclopropyl) in the sugar component, can result in the nucleosides being 'locked' in a specific conformation.

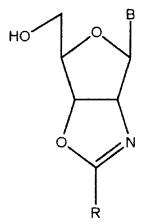

Figure 1

B = U, C R = H, alkyl, aryl

Methodology described by us for the synthesis of novel tetrahydrofuro[3,4-*d*]oxazoles (2,3-oxazole-D-ribofuranose) *i.e.* a fused ring system, has been applied to the synthesis of conformationally restrained nucleosides (Figure 1), with conformational analysis and antiviral screening providing data regarding drug/enzyme specificity.

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***In vitro* antiviral nucleotide analogs activation by cellular kinases and reaction with HIV reverse transcriptase : improvement by α-boranophosphate substitution**

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AIDS chemotherapy is usually limited by inadequate intracellular concentrations of the active triphosphate form of nucleoside analogs, leading to incomplete inhibition of viral replication and the appearance of resistant virus. Antiviral nucleoside analogs such as AZT and d4T are substrates of thymidine kinase, thymidilate kinase and nucleoside di-phosphate kinase. The triphosphate analog is substrate of the reverse transcriptase and blocks viral DNA elongation due to the lack for the 3'OH on the ribose moiety. The kinases of the phosphorylation pathway react differently with AZT and d4T. AZT-DP is a poor substrate for NDP kinase with a catalytic efficiency 10^4 times lower than dTDP. Monitoring the phosphotransfer by fluorescence, d4T-DP was found a better substrate than AZT-DP. Cocrystal analysis of NDP kinase complexed with dTDP or GDP have shown that the main anchor of the nucleotide is a hydrogen-bond network involving the 3'OH, protein residues and the O_7 of the β−phosphate. A change of the 3'OH, as found with AZT, disturbs this network. However, d4T-TP bound to the NDP kinase active site presents an unusual H-bond between the 3'CH and the O_7 of the α-phosphate explaining the better reactivity of d4T. One oxygen of the β-phosphate does not interact with any residues in NDP kinase and reverse transcriptase and represents a target for chemical modifications. The Rp diastereoisomer of α−boranotriphosphate d4T and AZT is 10 times more efficient with both enzymes and is more stable towards nucleases. Repair of blocked DNA chains by pyrophosphorolysis is significantly reduced in reverse transcriptases from drug-resistant virus. Even if the borano derivatives are slighty weaker substrates for TMP kinase, they are promissing compounds in antiviral therapies.

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Substituted *N*-Heteroaryl-*N'*-(2,6-difluorophenethyl)thiourea Derivatives as Human Immunodeficiency Virus Reverse Transcriptase Inhibitors: Synthesis and Structure-Activity Relationship

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We report the development of a new group of nonnucleoside reverse transcriptase inhibitors (NNRTIs). One of the most active congeners of this series, i.e. *N*-(5-chloro-2-pyridinyl)-*N'*-(2,6-difluorophenethyl)thiourea (GP-49), was found to inhibit human immunodeficiency virus (HIV) type 1 [HIV-1(III_B)] replication in MT-4 cells at a concentration of 1.8 nM. Comparable activity data were obtained in CEM cells and PBMCs. This compound was not toxic for the host cells up to the limit of solubility (76 μM). This class of compounds proved effective against different strains of HIV-1 (subtype B: III_B and subtype D: NDK), but not against the replication of HIV-2 (ROD). HIV-1 strains containing the L100I, K103N, Y181C, Y181I or Y188L mutations in their reverse transcriptase (RT) displayed reduced sensitivity to the compounds. From a structure-activity-relationship viewpoint, a number restrictions applied as to the chemical modifications that were compatible with anti-HIV activity. The *N'*-(2,6-difluorophenethyl)thiourea moiety was kept constant in the whole series and the heteroaryl substituents were varied. Substituted 2-pyridines and 2-pyrimidines were introduced. The pyridine congeners gave stronger inhibition of viral replication (100-fold) than the pyrimidines. The 5-brominated or -chlorinated pyridin-2-yls were the most active members of the group. Introduction of substituents at position 3 or 4 of the pyridine ring was detrimental to anti-HIV activity. Substituents (F, Cl, Br, Me) at position 6 did not affect the antiviral potency.

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Synthesis and Biological Activity of 1-Substituted Benzimidazole-2-thione Analogues: A New Class of Human Immunodeficiency Virus Reverse Transcriptase Inhibitors

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8-Chloro-TIBO and 1-(2,6-Difluorobenzyl)-2-(2,6-difluorophenyl)-4-methylbenzimidazole have been described as potent nonnucleoside reverse transcriptase inhibitors (NNRTIs). We have synthesized a series of hybrids, i.e. 1-substituted benzimidazole-2-thione analogues, e.g. 1-(2,6-difluorobenzyl)-7-aza-6-chlorobenzimidazol-2-thione (GP-38), which was found to inhibit human immunodeficiency virus (HIV) type 1 [HIV-1(IIIB) and NL4.3] replication in MT-4 cells and PBMCs at a 50% effective concentration of about 2 μM. Comparable activity data were obtained in CEM cells. The compound was not toxic for the host cells up to limit of solubility (80 μM). GPRTI-38 proved effective against a variety of HIV-1 strains (subtype B: IIIB, NL4.3 and subtype D: NDK), a clinical isolate L1 and a recombinant HIV-1 strain containing the nucleoside RT-characteristic mutations K70R, T215Y, 69SS, A62V, E79K, V118I, M184V, E203K, L210W, but not against the replication of HIV-2 strains ROD and EHO. HIV-1 strains containing NNRTI characteristic mutations in their reverse transcriptase (RT), i.e. K103N, displayed reduced sensitivity to the compounds. HIV-1-resistant virus containing the Y181C mutation in the RT was selected after thirteen passages of HIV-1(IIIB) in MT-4 cells in the presence of increasing concentrations of 1-(2,6-difluorobenzyl)benzimidazole-2-thione (GP-35). Of the 7-aza analogues, the 7-aza-6-chloro derivative displayed the highest selectivity. Introduction of 4-methyl or 4-methoxy substituents in the benzimidazole moiety lead to highly cytotoxic congeners.

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Antiviral and Biological Activities of 2-Amino-6-arylsulfonylbenzonitrile Analogues.

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Many highly potent non-nucleoside reverse transcriptase inhibitors (NNRTIs) of HIV-1 have been reported in the literature and three - nevirapine, delavirdine and efavirenz - have been approved by the FDA for use in combination therapy regimens. Although members of this class are of diverse chemical structures, they share the same binding motifs and inhibit the enzyme by binding in an "allosteric" site distinct from the catalytic site. Our screening efforts have identified a series of 2-amino-6-arylsulfonylbenzonitriles with nanomolar activity against HIV-1. One of the most potent compounds in the series, 739W94, had an IC_{50} value of 21.9 nM for HIV-1 and an IC_{50} value of 7 nM against HIV-1 reverse transcriptase. Structure-activity relationship studies have demonstrated that the addition of a lipophilic *meta* substituent to the 6-arylsulfonyl moiety significantly increases the antiviral activity. The presence of two *meta* substituents with one being a methyl group appeared to give the most optimal antiviral activity. These compounds generally retain potent antiviral activity when tested in a panel of clinically relevant mutant viruses where nevirapine was not effective. When tested in combination with other antivirals, the compound was found have synergistic activities with AZT and amprenavir and was additive to the activity of nevirapine and ddI. Additive or synergistic effects may indicate clinical efficacy in combination with current antiviral therapies.

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Hydrolysis Behavior of Benzyl-Functionalised *cyclo*Sal-d4TMPs

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*Cyclo*Saligenyl- (*Cyclo*Sal-) nucleotides have been developed to deliver nucleotides in cells selectively via chemical hydrolysis. Previously, only salicylic alcohols are formed as second product beside the nucleotide. In this work, we describe synthetic routes to new benzyl-functionalised *cyclo*Sal-d4T monophosphates (7-X-*cyclo*Sal-d4TMP; X = Me, *n*Bu, CH_2CO_2Et, CH_2SO_2Me, CH_2CN, CCl_3). These compounds have been prepared in order to prove the possibility of the formation of styrene-type elimination products instead of the above mentioned salicylic alcohols. The elimination products would be formed after a heterolytic benzyl C-O-bond cleavage and a subsequent proton-abstraction in the side chain.

The hydrolytic behavior of the phosphate triesters were studied both in a weakly basic aqueous solution (pH = 7.3) and in crude cell extracts. Their hydrolysis half lives as well as the product distribution were determined. These data were compared to the behavior of the corresponding *cyclo*Sal-d4TMPs without benzyl substituents. Surprisingly, a strong influence of the 7-benzyl substituent on the degradation pathway was found: For the first time two different degradation pathways were observed leading to different products. Cleavage of the benzyl C-O-bond led to phenyl diesters that did not further hydrolyze chemically. Nucleophilic attack of hydroxide to the phosphorus atom gave benzyl diesters and resulted simultaneously in an "Umpolung" of the *ortho*-phenyl substituent. By this "Umpolung" the intermediate benzyl diesters were able to further degrade to give d4TMP and the styrene type elimination product.

It was possible to exert influence on the ratio of the degradation products by further substitutions on the phenyl systems of the benzyl functionalised prodrugs. Evidences for two different degradation pathways have been found by HPLC-kinetics as well as by tandem-MS methods and will be discussed with the help of results of quantum mechanics calculations. The different anti-HIV activity of these compounds could be correlated with their different hydrolysis behavior.

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CADA: A Chemotherapeutic Compound that Inhibits HIV and HHV-7 Replication by Down-Modulation of the CD4 Receptor Expression

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Cyclotriazadisulfonamide (CADA) is a synthetic macrocycle with anti-HIV activity. CADA inhibited HIV replication with an IC_{50} ranging from 0.2-2.0 μg/ml when evaluated in different $CD4^+$ T-cell lines (such as MT-4 and SUPT-1) and in PBMC. It also inhibited HHV-7 replication in SUPT-1 cells and in PBMC with an IC_{50} ranging from 0.2-0.9 μg/ml. As both viruses use the CD4 receptor as the primary receptor to enter the cells, CD4 receptor expression was investigated in the presence or absence of CADA. Interestingly, this compound specifically down-modulated CD4 receptor expression within the same concentration range as it inhibited viral replication. Moreover, no effect was seen on other cell receptors such as CD2, CD3, CD5, CD8, CD26, CD28, CD38, HLA-DR or the chemokine receptors/HIV coreceptors CXCR4 and CCR5. Although phorbol esters (such as PMA) are the only other compounds described so far to down-modulate CD4 expression, CADA is clearly different in its mode of action. Whereas PMA already inhibits CD4 receptor expression within 1 hour, the effect of CADA becomes apparent only after 24 hours. The compound does not bind to the CD4 receptor directly. Nor does it inhibit virus binding. Preincubation of the cells with CADA for 24 hours improved its antiviral activity drastically. Interestingly, both HIV and HHV-7 also down-regulate CD4 receptor expression, and this down-regulation process is even further enhanced by CADA. In summary, CADA has a unique mode of action that it inhibits HIV and HHV-7 replication by down-modulating CD4 expression.

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In Vitro Antiviral Studies of the HIV-1 Nonnucleoside Reverse Transcriptase Inhibitor, Capravirine, Alone and in Combination with HIV-1 Protease Inhibitors.

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Inhibitors which target the reverse transcriptase (RT) of HIV-1 are an integral part of current therapeutic regimens. However, due to the rapid emergence of broadly cross-resistant HIV-1 variants in some patients, there is a pressing need for new antiviral agents. Capravirine (formerly AG1549) is a novel HIV-1 nonnucleoside reverse transcriptase inhibitor (NNRTI) that exhibits potent in vitro antiviral activity against both laboratory strains and clinical isolates, with EC_{50} values from 0.7 to 10.3 nM and EC_{90} values from 2.4 to 21.5 nM. Capravirine also maintains potent activity against HIV-1 strains that contain RT amino acid substitutions which have been shown to confer resistance to other NNRTIs and nucleoside reverse transcriptase inhibitors (NRTI). In support of its potential use in combination therapy regimes, the antiviral activity of capravirine alone and in combination with the protease inhibitors (PI), nelfinavir, ritonavir, indinavir, or saquinavir was evaluated in vitro against acute HIV-1 IIIB and HIV-1 RF infections of MT-4 and CEM-SS cells, respectively. The resulting antiviral effects were analyzed by the method of Prichard and Shipman and indicate that the combination of capravirine with each of the PIs tested resulted in strongly synergistic interactions. Results described here suggest that co-administration of capravirine with clinically approved anti-HIV-1 PIs may result in enhanced antiviral activity, in vivo.

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Activity of SJ-3366 a novel nonnucleoside reverse transcriptase inhibitor of HIV-1 in combination with other anti-HIV agents against wildtype and SJ3366-resistant viral isolates.

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The failure of single agent anti-HIV therapy is linked to the rapid replication and subsequent viral diversity of HIV which leads to the emergence of drug-resistant viral strains. Potent and rationally designed chemotherapy combining protease and reverse transcriptase (RT) inhibitors can dramatically slow the progression of HIV infection in patients. In the present study, SJ-3366, a nonnucleoside reverse transcriptase inhibitor which inhibits HIV-1 replication at concentrations below 1 nM and exhibits a therapeutic index of greater than 4,000,000 was evaluated in combination with protease inhibitors as well as other mechanistically diverse inhibitors of HIV-1. These combination assays were conducted against wild type (WT) and SJ-3366-resistant viral isolates. SJ-3366-resistant viruses were selected *in vitro* by exposing $HIV\text{-}1_{IIIB}$ to dose-escalating treatments of SJ-3366. Three SJ-3366-resistant isolates from the selection assay were utilized for the combination assays: HIV_{Y181C} (passage 4); $HIV_{E6E/K,\ V106V/I,\ Y181C,\ F227L}$ (passage 13); and $HIV_{E6E/K,\ V106I,\ Y181C,\ F227L}$ (passage 20). SJ-3366 was evaluated in two and three-drug combination regimens with zidovudine, didanosine, lamivudine, 2'-Fluoro-2',3'-dideoxyarabinosyladenine (F-ddA), nevirapine, efavirenz, indinavir, ritonavir, nelfinavir, ISIS 5320, resobene, Nab 2F5 (anti-gp41) and Nab 2G12 (anti-gp120). These combinations were also tested against $HIV\text{-}1_{IIIB}$ WT. Drug interactions were evaluated using the Prichard and Shipman MacSynergy II model. With WT virus, additive to slightly synergistic interactions were seen with most combinations, with some notable exceptions. The two-drug combinations of SJ-3366/didanosine, SJ-3366/F-ddA and SJ-3366/Nab 2G12 demonstrated considerable levels of synergy. In the combination assays using SJ-3366-resistant isolates, higher concentrations of SJ-3366 were required in order to compensate for the level of resistance to SJ-3366. Results for combination assays with SJ-3366-resistant isolates were similar to those for WT, however, the three-drug combination of SJ-3366, zidovudine, and nevirapine exhibited enhanced synergistic activity against these isolates. None of the combinations analyzed showed synergistic cytotoxicity.

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5-Arylthiopyrroles (ATPs): A new class of potent NNRTIs active against wild type and resistant strains of HIV-1.

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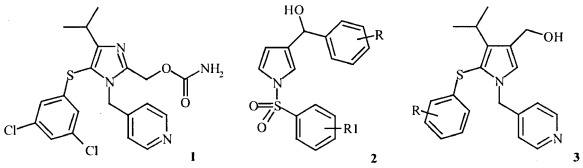
Despite the increased availability of therapeutic agents to treat infection with human immunodeficiency virus (HIV), there is an urgent need for new antiretrovirals to treat strains resistant to the currently used drugs. Recently, the imidazole derivative S-1153 (AG 1549) (**1**) has been reported which is endowed with potent anti-HIV-1 activity against a number of NNRTI-resistant HIV-1 strains, such as L100I, K103N, Y181C, Y188C mutants[1]. Our interest in pyrrole derivatives as potential chemotherapeutic agents has led us to design and synthesize a number of pyrrole derivatives such as **2**, with very potent anti-HIV-1 activity[2]. Pursuing those studies, we designed and synthesized 5-arylthiopyrroles (ATPs) **3,** structurally related to pyrrole derivatives **2** and S-1153. A number of these compounds showed potent HIV-1 activity at nanomolar concentrations, and were active against Y181C and K103R mutant viruses.



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NEW ANTIOXIDANT MOLECULES: ADJUVANT THERAPY AS TREATMENT OF OXIDATIVE DAMAGE CAUSED BY NRTI AND OF NEUROAIDS

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Several side effects of NRTI (neuropathy, myopathy, hepatotoxicity) could be attributed to oxidative damage generated by the inhibition of mitochondrial DNA polymerase γ. The dysregulation of cellular redox state may be also involved in neuroAIDS, and no specific therapy is currently available. The aim of this study was to evaluate *in vitro* new pro-glutathione (GSH) molecules as potential adjuvant therapy to counteract these deleterious process. Glutathione is the most aboundant intracellular antioxidant compound, and its level is decreased in HIV-infected patients and its oxidation correlated with mitochondrial DNA damage. In our experimental models (human lymphocytes and macrophages), the GSH pathway is disorganized in response to HIV infection (decreased GSH content, increased activity of GSH-Px), and a prodrug of NAC and MEA, I-152, demonstrated anti-HIV-1 activity (ED50 = 5 µM for 0.001 m.o.i.). This compound synergizes with AZT (CI < 1) and additive effects were observed with NNRTI and IP (CI = 1). Antioxidant and anti-inflammatory effects of I-152 included a restoration of intracellular GSH level (I-152-treated macrophages: 221 nmoles GSH/mg protein vs. untreated macrophages: 100 nmoles GSH/mg protein) and a decrease of TNF-α synthesis in LPS-stimulated monocytes/macrophages (I-152-treated cells: 17 ng/ml vs. untreated cells: 8.75 ng/ml). Altogether, these *in vitro* results suggest that this new GSH-replenishing drug could be beneficial as therapeutic agent in HIV-1-infected patients, for treatment of NRTI side effects and of neuroAIDS.

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Inhibition of HIV-1 Replication and Integrase Activity by a Series of Diketo Derivatives

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Recently L-708,906, a diketo acid derivative, has been described as a selective inhibitor of HIV-1 integration (Hazuda *et al.*, Science 287: 646-50, 2000) while 5ClTEP, another diketo derivative, has been co-crystallized with the core domain of HIV-1 integrase (Goldgur *et al.*, Proc. Natl. Acad. Sci. USA 96: 13040-3, 1999). Here we present a comparative evaluation of these compounds and other, newly synthesized diketo analogues. All compounds tested inhibited HIV-1 integrase activity. L-708,906 and a chimeric molecule, P13, incorporating the diketo acid moiety into 5ClTEP were very potent inhibitors of the global integration process. They inhibited both 3'-processing and DNA strand transfer. Both L-708, 906 and P13 were shown to compete with the target DNA substrate. However, of all the diketo analogues tested, only L-708,906 was found to inhibit HIV-1(NL4.3) replication in MT-4 cells and PBMCs, at a 50% effective concentration of 25 and 16 µM, respectively. The compound was not toxic for the host cells up to the limit of its solubility (62 µM). L-708,906 proved effective against a variety of HIV-1 strains (subtype B: III_B, NL4.3 and subtype D: NDK), a clinical HIV-1 isolate L1, different strains of HIV-2(ROD and EHO) and SIV(MAC_{251}). L-708,906 was equally active against virus strains that were resistant towards polyanionic and polycationic types of viral entry inhibitors as well as virus strains selected for resistance against nucleoside and nonnucleoside reverse transcriptase inhibitors. Time-of-addition experiments indicated that the compound interfered with the viral replication cycle at a time point (more than 7 hours post-infection) coinciding with the time of retroviral DNA integration. In conclusion, L-708,906 can be considered as a reference compound in the evaluation of authentic integrase inhibitors.

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Development, Optimization and Application of a Cell-Based Assay to Identify Inhibitors of HIV-1 Integrase

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Integration of HIV-1 DNA into the host genome is an essential step in HIV-1 replication and represents an important potential target for design of anti-HIV-1 integrase (IN) agents. The integration event requires two separate reactions; 3'-end processing of the LTR terminus or donor substrate, and a DNA strand transfer step. In vitro assays currently exist for screening inhibitors of either biochemical reaction step, however recent studies suggest only inhibitors of the strand-transfer reaction are specific for HIV-1 integrase in vivo. We have extended the existing *in vitro* anti-IN screening assays by developing a cell-based assay to evaluate promising anti-IN agents within the context of the cellular milieu. This approach involves a combination of quantitative real-time PCR and anchored/nested PCR to quantify levels of unintegrated pre-integration complexes (PICs) and integrated proviral DNA, respectively. Current utility of this approach has been demonstrated with the characterization of chicoric acid and several analogs as anti-IN inhibitors; future studies with 5-Cl-TEP and the Merck diketo acids will provide additional validation for this experimental approach. As a final evolution in the development of cell-based anti-HIV-IN assays, we will present several modifications to the assay which make the assay amenable to larger-scale mid-to-high throughput screening efforts.

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Molecular Modeling of Inhibitors in the Active Site of HIV-1 Integrase

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Integration of proviral DNA into the host DNA is a critical step in the life cycle of the Human Immunodeficiency Virus (HIV). The reaction is catalyzed by a viral enzyme, integrase. The two main reactions catalyzed by integrase are 3'-processing and strand transfer. Inhibitors of either of these processes may be expected to afford leads to HIV therapeutics with modes of action different to those presently available. Although an X-ray crystal structure of the core domain of HIV-1 integrase has been available for several years[1] a clinically proven integrase inhibitor is yet to be developed. We have utilized a recent X-ray crystal structure of the HIV-1 integrase core domain containing a bound inhibitor (5ClTEP) and active site magnesium ion.[2] Docking (AUTODOCK)[3] and molecular dynamics studies have been performed on a range of literature inhibitors of HIV-1 integrase for which biochemical data suggests a binding mode at or near to the catalytic active site. These studies highlight the importance of not only the active site magnesium ion but also its solvation shell that we propose forms part of the 5ClTEP binding pocket. Results from docking experiments of 5ClTEP and a number of other inhibitors will be presented. Docking studies on several literature compounds, employing a similar approach, have recently been published[4] and comparisons to this work will be made.

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HIGH THROUGHPUT ASSAY FOR SCREENING ANTI HIV-1 FUSION, INTEGRASE OR REVERSE TRANSCRIPTASE INHIBITORS. S. J. Fleming, J. P. Mewshaw, A. Lal and L. T. Rimsky. Triangle Pharmaceuticals Inc. 4611 University Drive, P.O. 50530 Durham NC 27717-5030. USA

Treatment of HIV-1 infected patients with reverse transcriptase or protease inhibitors has resulted in an increased number of patients carrying HIV-1 viruses resistant to all commercially available anti-HIV-1 drugs. The development of compounds targeting entry, fusion or integration of HIV-1 is in progress. Nevertheless, there will be an increasing need for a variety of compounds targeting these and other steps of the viral replication, as they too, will induce selection for drug resistant viruses over time. Therefore our objective was to develop a high throughput screening assay of anti-HIV-1 compounds involving minimal human labor and high reproducibility. The assay described in this study was named MAGI-LU assay. It is based on the previously described MAGI cells (J. Virol.1992. 66:2232-2239) assay. The MAGI assay exploits the ability of the Tat protein of HIV-1 to transactivate an integrated β-galactosidase gene driven by the HIV-1 LTR in CD4 transfected HeLa cells. HIV-1 infected MAGI cells specifically express β -galactosidase that can be detected by visualization of blue colored cell nuclei. The blue nuclei are counted to determine the level of infectivity. Conversely, in this assay the β -galactosidase is detected using a commercially available chemiluminescent reporter gene assay (Gal-Screen™, Tropix). Our results present an adaptation of the MAGI assay that give a numeric signal directly proportional to the amount of virus present in the culture therefore leading to true IC_{50} values. The MAGI-LU assay proved to be highly reproducible within one assay and from assay to assay. We observed that the reproducibility varies by no more than a factor of 2. This assay is easily adaptable to a robotic system that delivers high throughput compound screening analysis. In conclusion the MAGI-LU assay proved to be a non-labor intensive, cost effective, and highly reproducible assay with optimal performance in automated high-through-put screening. Complete phenotypic analysis of the hits can be done with the MAGI-LU assay.

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Alterations In Virus Fitness And Gag-Pol Processing By Specific Resistance Engendering Protease Mutations.

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Impaired virus fitness and abnormalities in gag-pol processing may explain the stable CD4+ counts in patients harboring protease-resistant virus strains. Therefore, we evaluated the effects of single protease inhibitor mutations G48V and L90M and of the dual mutation G48V/V82A on virus fitness and processing of Gag precursors by HIV-1 protease. Individual virus replication and competition assays with wild type virus were performed by transfection of equal amounts of mutant and/or wild type DNA into CEM-SS cells. The resulting infections were analyzed over the course of infection by both reverse transcriptase production and population based sequencing. Mutant and wild type processing of Gag precursors were evaluated in transfected HeLa cells by immunoprecipitation of [^{35}S] labeled HIV-1 proteins 48 H post-transfection. The single-mutation G48V and the dual-mutation G48V/V82A demonstrated an approximate two-fold reduction in virus replication with a 6 to 10 day delay before peak virus replication compared to WT virus or the L90M mutant. The L90M replicated with similar kinetics and efficiency as WT, with peak virus production delayed by two days. Interestingly, the WT virus was out-competed by the L90M mutant virus in the cultures by day 6, indicating a higher level of replication fitness. Conversely, the WT virus out-competed the G48V mutant in the cultures by day 9 and the G48V/V82A by day 12. Preliminary results indicate that protein processing by these protease mutant strains was abnormal. All the mutants displayed additional bands not detected in the WT indicating incomplete cleavage of the Gag precursor proteins. Understanding the effects of each protease mutation, alone and in combination, may prove valuable in selecting treatment options for HIV-1 infected patients.

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Anti-HIV activity of a neomycin B-arginine conjugate

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We have previously described conjugates of L-arginine with aminoglycosides (AAC) that have shown anti-human immunodeficiency virus type 1 (HIV-1) activity in *in vitro* cell culture systems. Here, we extend our report to a novel neomycin B-arginine conjugate (NeoR) that has shown up to 30-fold increased potency over previous AAC compounds. NeoR inhibited the replication of both R5 and X4 strains of HIV-1 in cells expressing the appropriate coreceptor or peripheral blood mononuclear cells. In lymphoid tissue ex vivo, NeoR blocked the replication of the dualtropic strain 89.6 suggesting anti-HIV activity of AAC on the site of *in vivo* virus replication. NeoR blocked the binding of HIV particles to lymphoid cells and was also able to antagonize the activity of the CXCR4 receptor so it may prevent the emergence of X4 HIV-1 strains. Nevertheless, we were unable to detect anti-Tat dependent transactivation activity as previously suggested for this family of compounds.

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Design and Synthesis of Peptide Libraries as Lead Compounds for Specific Cell-Virus Interaction Inhibition, using Solid Phase Combinatorial Chemistry.

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HIV has cost a huge number of lives over the last 20 years and the epidemic is still spreading. In 1998, the number of people living with HIV increased by 10%. A number of drugs have been developed to treat HIV infection; these include Reverse Transcriptase Inhibitors and Protease Inhibitors. Unfortunately, the virus rapidly develops resistance to these drugs limiting their therapeutic utility. The use of combinations of inhibitors has proved much more successful in the treatment of HIV infection. However, there is still possibility of resistance even for this combination therapy. Therefore, there is an urgent need for the development of new agents for the treatment of HIV infection, particularly agents acting on new molecular targets.

One particularly attractive target is to prevent the fusion between the viral and the human cell membranes, which will prevent entry of the virus into the cell and incorporation of the viral genome into the human genome. A key protein for this process is the protein gp120 on the HIV surface, which interacts with the CD4 receptor and chemokine receptors on the target cell. These interactions result in conformational changes in the viral proteins, exposing a hydrophobic fusion domain allowing fusion of viral and cell membranes.

The work presented here is on the design, synthesis and evaluation of peptides, which may prevent interaction between HIV and the cell. Small peptides, which prevent such events, may represent drug leads.

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Anti-HIV-1 Activity of A New Class of Oligonucleotide Trgeted to the Polypurine Tract of Viral RNA

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The PPT is highly conserved among the known HIV-1 strains, and is a possible target for triplex formation. We show the effects of triple-helix formation by assays of RNase H cleavage inhibition *in vitro*, using a two-strand-system (FTFOs, DsDGloopT5-37) targeted to the polypurine tract (PPT) of HIV-1. The two-stranded composition of a triple-helix is thermodynamically and kinetically superior to the three-strand-system. The FTFOs inhibited the RNase H activity in a sequence-specific manner, i.e., the triplex actually formed at the PPT and blocked the RNase H. The FTFOs containing the phosphorothioate groups at the antisense strand showed greater 3'-exonuclease resistance. In the observation of the FITC-labeled-FTFO in MOLT-4 cells by a confocal laser scanning microscope, diffuse fluorescence was apparently observed in the cytoplasm and nucleus. However, weak fluorescence was observed within the cytoplasm and nucleus of MOLT-4 cells treated with the antisense phosphorothioate oligonucleotides. In HIV-1 infected MOLT-4 and PBM cells, the FTFOs containing the phosphorothioate groups at the antisense strand and guanosine rich parts within the third Hoogsteen base pairing sequence inhibit the replication of HIV-1 more effectively than the antisense phosphorothioate oligonucleotides, indicating sequence-specific inhibition of HIV-1 replication in long term assay (60 days). We also describe the mechanism of anti-HIV-1 by the FTFOs

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Iron chelators in antiviral therapy

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Combinations of the iron chelator deferoxamine (DF) with the oral chelator deferiprone (L1) reportedly had a beneficial effect in iron excretion in iron overloaded thalassemic patients. We wanted to investigate whether the same was true in relation to HIV-1 inhibition where two mechanisms of inhibition by iron chelators have been proposed: (i) cellular proliferation inhibition (ii) NF-κB inhibition. We have shown earlier that non-toxic concentrations of either DF or L1 when used as single agents significantly lowered HIV-1 replication by inhibiting cellular proliferation in human peripheral blood lymphocytes (PBL) (30 μM DF and 100 μM L1 resulted singly in 85% HIV-1 inhibition and proliferation inhibition). We investigated whether the combination DF-L1 synergistically or additively inhibited HIV-1 in PBL, and whether the mechanism of inhibition, if any, was via cellular proliferation inhibition. In two of the three donor PBL the combination DF-L1 was synergistic and in the third slightly additive. In one experiment, the IC_{50} values of the two drugs when used alone were 14 and 62 μM respectively. More than 100x less of either DF or L1, respectively were needed when in combination to achieve 50% HIV-1 inhibition. At these low concentrations of the two drugs the decrease in cellular proliferation could not fully account for the HIV reduction. Combinations of the two drugs in transfected Jurkat cell lines expressing the HIV-LTR plasmid coupled to a luciferase reporter gene also resulted in synergistic inhibition of luciferase production, implying that iron chelators when used in combination, could also inhibit HIV-1 via proviral transcription inhibition.

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INFLUENCE OF CHARGE DISTRIBUTION ON THE ANTI-HIV ACTIVITY OF NEGATIVELY CHARGED ALBUMINS.

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Increasing the net negative charge of proteins results in agents with potent anti-HIV activity. Previously, the antiviral properties of albumin modified by succinylation (Suc-HSA) and aconitylation (Aco-HSA) were reported. The anti-HIV effects were based on an inhibition of HIV-cell binding and subsequent fusion, through interaction of these agents with HIV gp120 (V3 loop). The *in vitro* IC_{50}-values for Suc-HSA and Aco-HSA were 5 and 0.5 nM, resp. We now studied whether the distribution of the negative charges on albumin is essential for the observed anti-HIV effect. Therefore, we prepared albumins with clusters of negative charges and assessed *in vitro* the anti-HIV IC_{50} and the binding to V3 loop. Also, the *in vivo* behaviour of negatively charged albumins was studied in rats.

Clustering of negative charges on albumin was obtained by covalent coupling of heparin at various chain lengths (Hep). Three batches were obtained with increasing size of Hep chains: Hep3kD-HSA, Hep6kD-HSA and Hep13kD-HSA. All batches were primarily in the monomeric form and displayed a strong negative charge (pI<3). Hep6kD-HSA and Hep13kD-HSA were active against HIV pathogenicity (IC_{50} = 0.66 and 0.15 μM, resp.), in contrast to HSA modified with Hep3kD. HSA with an increased extent of Hep13kD substitution displayed improved anti-HIV activity (IC_{50} =0.02 μM). Further substitution of Hep13kD-HSA with Aco-groups resulted in even lower IC_{50}-values (0.002 μM). Similar to Suc-HSA, Hep13kD-HSA and Hep6kD-HSA bound to V3 loop peptides, although to a lesser extent than Suc-HSA did. These negatively charged albumins displayed no cytotoxicity. *In vivo*, at 10 min after iv injection of Hep13kD-HSA (tracer) into rats, the protein was detected in blood (35%), liver (56%) and spleen (1.3%). A dose of 4 mg/kg resulted in constant plasmaconcentrations for at least 2 hours (0.1 mg/ml). Additionally, it was shown that Hep13kD-HSA distributes to the lymphatic system, reaching concentrations that exhibit antiviral effect *in vitro* in this compartment.

In conclusion, Hep13kD-HSA with clustered negative charges showed anti-HIV activity by binding to gp120 V3 loop. However, random modification of HSA with negatively charged groups, as obtained by succinylation of HSA (Suc-HSA), resulted in more potent anti-HIV activity *in vitro*. On the other hand, *in vivo* studies show longer plasma $t_{1/2}$ and higher concentrations in the lymphatics (important HIV reservoirs) for Hep-HSA. These kinetic features may favour anti-HIV effect *in vivo*.

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Studies on Antiviral Compounds-Synthesis of the Natural Stilbene-Pinosylvin and its derivative

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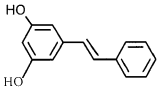
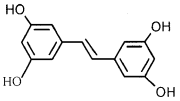
We have synthesized two natural products resveratrol (Ⅰ,(E)-3,5,4'-trihydroxy - stilbene) and isorhapontigenin (Ⅱ, (E) - 3,5,4'-trihydroxy –3'-methoxy-stilbene) which have been isolated from *G.parvifolium* , a traditional Chinese herb used to treat rheumatoid arthritis, ulcer bleeding and bronchitis . The preliminary anti-HIV test showed that I had anti-HIV activity in H_9 cell with an IC_{50} of 9.79uM. Pinosylvin (III, 3,5-dihydroxy-stilbene) is another natural hydroxyl stilbene isolated from this plant. We consider that the structure of (Ⅲ)is similar to (Ⅰ)which a hydrogen at the 4'-position instead of hydroxyl group and so we prepared pinosylvin Ⅲ and its derivative (IV, 3,5,3',5'-tetrahydroxy stilbene) which two hydroxyl groups at the 3', 5'- position instead of 4'- hydroxy group by seven and eight step reaction sequence respectively in order to test their anti-HIV as well as their anti-HIV reverse transcriptase activity. The antiviral evaluation is undergoing .



pinosylvin III



IV

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Cross-resistance is not the end of the NNRTI story.

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A current paradigm is that resistance to NNRT inhibitors can be readily acquired and results in high-level resistance to the entire class of drugs. In order to be useful, new NNRTIs need to be active on single and double NNRTI mutants, and achieve acceptable trough plasma levels in patients. To identify metabolically stable compounds that are active on NNRTI resistant strains, we applied a parallel screening strategy, including antiviral assays using wild type and resistant HIV strains, and metabolic stability assays. Optimisation of a lead structure by simultaneous analysis of structure-activity and structure-metabolism relationships led to the identification of R165335-TMC125, a diaminopyrimidine (DAPY) derivative, with an antiviral potency in the nanomolar range against both wt and resistant strains (e.g. IC_{50} for K103N+Y181C double mutant = 4 nM). The compound is stable in human liver microsomes (< 20% degradation), is not toxic in MT4 cells (CC_{50}>100μM), and its IC_{50} does not increase significantly in the presence of human serum proteins. Development of in vitro resistance to the drug can completely be prevented at a 1μM concentration, when using a very high MOI. A total of 1,081 NNRTI resistant recombinant clinical isolates were phenotyped according to the Antivirogram™ method. The percentage of these with an IC_{50} of 100 nM or lower was 54 % for efavirenz, against 97 % for R165335-TMC125. This new NNRTI has a unique resistance profile, and exhibits very little cross-resistance to other inhibitors of this class.

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Resistance and Cross-Resistance Profiles of Capravirine, A Novel Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI) of HIV-1

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The emergence of HIV-1 variants resistant to all current antiretroviral agents in some patients necessitates the development of new reverse transcriptase inhibitors (RTI), particularly those that can be utilized for NNRTI-treatment experienced patients. Capravirine (formally AG1549) is a novel HIV-1 NNRTI that exhibits potent *in vitro* antiviral activity against both laboratory strains and clinical isolates. Previous studies have described the isolation of HIV-1 IIIB variants containing the V106A/F227L and K103T/V106A/L234I substitutions after prolonged passage in the presence of increasing concentrations of capravirine (Fujiwara et al., 1998, *Antimicrob Agents Chemother.* 42:1340-5). We have further evaluated the resistance and cross-resistance profiles of capravirine through selection of resistant HIV-1 variants from *in vitro* serial passage of additional HIV-1 strains in the presence of increasing concentrations of capravirine. The HIV-1 variants selected contained multiple substitutions in diverse patterns including L100I, Y181C, G190E and/or L234I combined with one or more of 20 different NNRTI-associated substitutions. Profiles of susceptibility to capravirine or other NNRTIs for HIV-1 variants engineered to contain capravirine resistance-associated substitutions ranged from hypersusceptible to highly resistant. Alternatively, capravirine demonstrated potent activity against recombinant HIV-1 strains derived from the plasma of antiretroviral-treatment experienced patients and that contained NNRTI- and NRTI-resistance associated substitutions. Full susceptibility to capravirine was observed for isolates containing K103N, L100I/K103N, or K103N/P225H, mutations frequently associated with broad cross-resistance to other NNRTIs. Results from these studies describe a unique resistance profile for capravirine and suggest the potential for capravirine antiviral efficacy in NNRTI-treatment experienced patients.

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Broad-spectrum inhibition of HIV-1 resistant mutants by novel indolyl aryl sulfones.

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In the search for new non-nucleoside reverse transcriptase inhibitors (NNRTIs) endowed with broad spectrum activity against mutant viruses resistant to other NNRTIs, we synthesized a series of novel indolyl aryl sulfones.

The new derivatives were evaluated against wt, Y181C, K103R and the double mutant K103N/Y181C. Nevirapine and Efavirenz were used as reference inhibitors. Within the structure-activity relationship, two indole derivatives were discovered that inhibited wild type, the two single mutants, and the double mutant in the nanomolar range.

These broad spectrum indoles are characterized by the presence of both a 3,5-dimethyl substituent on the phenyl ring and an amide function, either free or N-substituted, at position 2 of the indole. Substitution of the amide with an hydrazide group, while not compromising the potency against wt and Y181C strains, lead to loss of activity against the double mutant. These results suggested that activity of this class of indole derivatives against the double mutant K103N/Y181C depends upon the design of the phenyl ring and the substituent at position 2 of the indole ring.

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Effect of C-2 dialkylamino substitutions on DABOs antiviral activity spectrum against resistant mutants.

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In previous studies we evaluated the activity of representative S-DABO and N-DABO derivatives against clinically relevant HIV-1 resistant mutants. Most DABOs inhibit the Y181C and the K103R mutants but not the double mutant K103N/Y181C mutant. Structure-activity relationship studies suggested that the activity against resistant mutants correlateed with: i) the presence of two methyl groups at position 5 of the pyrimidine ring and on the methylene linker; ii) an appropriate substituent at position 2 of the pyrimidine ring.

In the present study we report a novel series of DABO derivatives characterized by replacement of the C2-alkylthio/alkylamino side chain with the isoster C2-dialkylamino side chain. Our aim was to establish a correlation between the chemical nature of the C2 substituent and the DABO spectrum against resistant mutants.

The introduction of new dialkylamino side chains leads to potent derivatives with an activity spectrum against resistant mutants partially different from that observed in previous series. Like S/N-DABOs, the new derivatives inhibit the single mutants Y181C and K103R with a potency related to the presence of the two methyl groups both at position 5 and on the linker. However, unlike S/N-DABOs, these new derivatives have markedly improved potency against the double mutant K103N/Y181C. Interestingly, the most potent derivative lacks the methyl groups at position 5 and on the methylene linker. This suggests that the requirements of N,N-disubstituted DABOs for activity against the double mutant are different than those necessary for activity against wild type and single mutants.

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

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Cellular factors have been proposed to contribute to the waning efficiency of chemotherapy. To gain new insights into this phenomenon, cellular lines resistant to different nucleoside analogues (NA) have been developed. Specifically cells resistant to antiviral activity of 3TC (CEM3TC) and d4T (CEMd4T) were obtained. Kinetics experiments performed to determine the intracellular accumulation of NA indicate that , at each point tested (1, 2, 4 hours), in resistant line there is a reduction ($p<0.005$) of the drug concentration inside the cells. In order to identify the mechanism/s underlying this phenomenon enzymatic assays were performed to evaluate the activity of cytidine kinase (CK) and thymidine kinase (TK) in CEM3TC and in CEMd4T respectively. The results indicate that in the d4T resistant line the TK activity is reduced as compared to the parental line (CEM). On the contrary, in CEM3TC cells, the enzymatic activity of CK is similar to control cells suggesting that the changes leading to drug resistance are different. Efflux study performed on CEM3TC, indicated that these cells have an increased capability to expel the drug (30%) compared to CEM (15%). In order to evaluate whether an ABC protein can be involved in the efflux of 3TC, the expression of Pgp, MRP1 and MRP4 in CEM and CEM3TC is under study. These data suggest that the phenomenon of cellular resistance to antiviral drugs may be due to two main mechanisms: enzymatic defect and/or an increased capability of efflux of antiviral drugs.

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Selection and characterization of HIV-1 variants resistance to a novel RT inhibitor BCH-13520

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Appearance of drug resistance is a major problem for therapy of human immunodeficiency virus type 1 (HIV-1) infection. BCH-13520 is a novel anti-HIV-1 agent which acts on reverse transcriptase (RT). To study potential HIV-1 resistance to BCH-13520, we passed $HIV\text{-}1_{IIIB}$ in the presence of gradually increasing concentrations of BCH-13520 in MT-2 cells to generate drug resistant variants. Genotypes of the selected virus were determined through directly sequencing a PCR-amplified fragment containing the RT coding sequence. A valine to isoleucine mutation at residue 75 (V75I) of the reverse transcriptase was observed at passage 11. Following further selection, the percentage of V75I mutated variant versus wild type virus gradually decreased and eventually completely disappeared. At the same time a variant carrying a lysine to arginine substitution at residue 65 (K65R) of RT was observed by passage 14. The K65R mutation remained upon further selection. These *in vitro* selected variants had an approximately 3-fold decreased sensitivity to BCH-13520 tested in MT-2 cells. V75I or K65R mutated HIV-1 RTs had altered recognition of deoxynucleotide substrates and dideoxynucleotide inhibitors. Currently, we are characterizing these mutated HIV-1 variants. The obtained results indicate that V75I and K65R mutations encode a low level of HIV-1 resistance to BCH-13520.

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Antiviral Resistance to SJ-3366, A Novel Dual Mechanism of Action Inhibitor of HIV-1 and HIV-2

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The anti-HIV compound SJ-3366 inhibits the replication of both HIV-1 and HIV-2 through specific inhibition of the reverse transcriptase of HIV-1 and inhibition of envelope mediated fusion events with HIV-1 and HIV-2. Against HIV-1, acting as a typical nonnucleoside reverse transcriptase inhibitor, SJ-3366 exhibits IC50 and IC95 concentrations in the low nanomolar to sub-nanomolar range. Inhibition of HIV-1 and HIV-2 entry occurs at concentrations two logs higher. In order to further delineate the role of the viral RT and env as the specific antiviral targets of SJ-3366, drug resistant strains of both HIV-1 and HIV-2 were selected in cell culture and the amino acid changes in RT and env were identified. With both viruses, the selection of resistant strains in cell culture was rapid and strains of virus which were completely insensitive to SJ-3366 were selected, requiring approximately 20-passages in culture for HIV-1 and 5 passages for HIV-2. With HIV-1, we have shown that the initial passages of virus yielded resistant strains that were no longer susceptible to the attachment inhibition mechanism of action of SJ-3366. This resulted in a strain of virus with approximately 1000-fold loss in sensitivity to SJ-3366. A variety of amino acid changes were detected in the envelope of HIV-1 during this early selection history (passages 1-5). The first RT specific mutation to occur was the typical Y181C amino acid change conferring NNRTI resistance. With increasing passage, additional amino acid changes in the hydrophobic NNRTI binding site were detected. At later passages the overall level of resistance of the virus to SJ-3366 continued to increase in the absence of identifiable mutations in RT. New amino changes did appear, however, in the envelope. We believe these changes may by "fitness" changes that allow the virus to replicate efficiently with the large number of accumulated mutations in RT and env. With HIV-2, resistance also appears rapidly and no amino acid changes are detected in the viral RT, as would be expected since SJ-3366 is a HIV-1 specific inhibitor of RT. Several amino acid changes do appear in the env. A comparison of the amino acid changes between HIV-1 and HIV-2 should yield information regarding critical changes in the env.

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Rapid Phenotypic Drug Susceptibility Assay for HIV Clinical Isolates.

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With the advent of HAART therapy for the treatment of HIV infected patients and its subsequent failure in some patients, there is a need for rapid phenotypic drug susceptibility testing. Currently available phenotypic drug susceptibility assays are too time consuming and labor intensive to be clinically relevant. The homologous recombination assays only monitor mutations in the protease and reverse transcriptase (RT) genes missing mutations that occur outside of these genes that may render antiretroviral therapy less effective. To remedy this situation, we developed a rapid, phenotypic drug susceptibility assay for HIV clinical isolates that utilizes genetically engineered cell lines that express green fluorescent protein (GFP) when infected with lymphotropic, macrophage tropic, or dual tropic HIV laboratory strains and clinical isolates. Ghost cells were obtained from the NIH AIDS Research and Reference Reagent Program. The cells were infected with $HIV\text{-}1_{LAI}$, $HIV\text{-}1_{LAI\text{-}M184V}$, and three clinical isolates in the absence and presence of various concentrations of AZT, 3TC, and efavirenz. After incubation for 48 hr, the cells were harvested and the percent of cells expressing GFP was determined by flow cytometry. The IC_{50} values for wild type virus were 0.07μM AZT, 2 μM 3TC, and 1.56 nM efavirenz. The 3TC resistant HIV isolate had IC_{50} values of 0.1 μM for AZT and >40 μM for 3TC. Three drug susceptible clinical isolates had IC_{50} values for these drugs similar to those of the wild type virus. Equivalent results were obtained with these three drugs when the ghost cells were infected with H9 cells chronically infected with HIV_{IIIB}. These results demonstrate the use of this rapid, phenotypic drug susceptibility assay for determining IC_{50} values for RT inhibitors of HIV replication. The assay should be particularly useful for drugs that block early events in the HIV replication cycle, such as compounds that block virus attachment.

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Development, Optimization and Application of Methodology to Assess Relative Replication Fitness of Reverse Transcriptase Inhibitor Resistant Viruses

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The rapid selection of drug-resistant strains during antiviral therapy of HIV infected patients is the primary reason for treatment failure with both nucleoside and nonnucleoside RT inhibitors. Although resistance would appear to be detrimental to antiviral therapy, it may be possible to select for resistant strains with amino acid changes that result in reduced replication capacity of the virus. Mutations that engender drug resistance may actually handicap the viral enzymes, reducing the rate and/or extent of virus replication. This reduced rate of virus replication in a patient may prolong the interval between HIV infection and AIDS, allowing the immune system to more effectively deal with the virus. Our laboratory has developed an assay to evaluate and compare the replication kinetics of wild-type NL4-3 virus with viral constructs containing specific amino acid changes in the reverse transcriptase. Cell line, cell density, DNA concentration, transfection methodology and efficiency were optimized to measure the rate and extent of virus replication in CEM-SS cells. Virus containing point mutations L74V, L100I, T215Y, and G190E, which confer drug resistance to nucleoside and nonnucleoside RT inhibitors, demonstrated reduced rate and extent of virus replication. K103N, M184V, Y188C, and Y188H grew equivalent to wild-type NL4-3. Several mutants (T139I, Q151M, Y181C, G190A, F227Y) grew slightly better than wild-type. Virus replication competition assays were performed by mixing equivalent amounts of wild-type NL4-3 with viruses possessing either L74V, K103N, Y181C, M184V, Y188C, Y188H, G190E, or Q151M. Relative growth potential of the mutant virus was compared to wild-type in the absence and presence of drug selective pressure. These results demonstrate that drug-induced mutations in the reverse transcriptase have both positive and negative effects on the ability of HIV to replicate in human cells. Further evaluation of each mutation, alone or in combination, may prove valuable in designing therapeutic strategies for HIV infected patients.

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Synergistic Anti-HIV Activity of DAPD in Combination with the IMPDH Inhibitors Mycophenolic Acid and Ribavirin.

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DAPD, (-)-β-D-2,6-diaminopurine dioxolane, is a selective inhibitor of HIV-1 replication *in vitro*. DAPD is deaminated *in vivo* by adenosine deaminase to give (-)-β-D-dioxolane guanine (DXG) which is subsequently converted to the corresponding 5'-triphosphate (DXG-TP). Biochemical analysis has demonstrated that DXG-TP is a potent inhibitor of the HIV-RT with a Ki of 0.019 uM. Mycophenolic acid (MPA) and ribavirin reduce the rate of de novo synthesis of guanosine nucleotides by inhibition of inosine monophosphate dehydrogenase (IMPDH). Reduction of intracellular dGTP levels through inhibition of IMPDH may effectively increase the intracellular concentration of DXG-TP thereby augmenting inhibition of HIV replication. We analyzed the effect of various concentrations of MPA and ribavirin on the antiviral activity of DAPD and DXG against wild type and mutant strains of HIV-1 in PBMC and in the laboratory adapted T-cell line MT2. When tested against wild type HIV-1 in MT2 cells, both MPA and ribavirin decreased the apparent EC_{50} for DXG by 10.5 and 12-fold respectively. An even greater effect on the activity of DXG was observed with these combinations in PBMCs. Addition of 0.25 μM MPA or 20 μM ribavirin completely inhibited virus replication at all concentrations of DAPD tested. HIV-1 isolates containing the L74V, K65R, or Q151M mutations are 4 to 10-fold less sensitive to inhibition by DXG than wild type virus. Addition of 0.25 μM MPA or 20 μM ribavirin completely reversed the DXG resistance observed with these isolates. The combination of 0.25 μM MPA or 20 μM ribavirin was not cytotoxic to the cells in these assays. In addition, when tested at physiologically relevant concentrations, neither compound demonstrated mitochondrial toxicity; alone or in combination with DAPD or DXG. These data suggest a potential role for the use of IMPDH inhibitors in combination therapy with DAPD in the treatment of HIV.

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Three-Drug Combinations of Emivirine (EMV), d4T and 3TC versus EMV, d4T and ddI in vitro: Long-Term Culture of HIV-1-Infected Cells and Breakthrough Viruses

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Emivirine (EMV, formerly MKC-442) is a nonnucleoside reverse transcriptase inhibitor (NNRTI) currently undergoing Phase III clinical trials in patients with EMV + d4T + 3TC and EMV + d4T + ddI. In this study, we examined the anti-HIV-1 activity of these three-drug combinations in cell cultures. MT-4 cells were infected with HIV-1 (III_B strain) and cultured in the presence of various combinations of compounds. The anti-HIV-1 activity of compounds was determined by the inhibition of HIV-1-induced cytopathicity, and the median-effect principle with isobologram method was applied for the multiple drug effect analysis. Combinations of EMV and two nucleoside RT inhibitors, either d4T + 3TC or d4T + ddI synergistically inhibited HIV-1 replication in MT-4 cells. The combination of EMV + d4T + 3TC showed higher synergism (smaller combination index) than that of EMV + d4T + ddI, irrespective of the endpoints used for synergy calculation. Higher synergism was also observed with the three-drug combinations than with any two-drug combinations, such as EMV + d4T, EMV + 3TC, EMV + ddI, d4T + 3TC, or d4T + ddI. When the occurrence of viral breakthrough was investigated during a long-term culture of infected MT-4 cells, both of the three-drug combinations ($10 \times EC_{50}$) completely suppressed HIV-1 replication at least for 40 days. In contrast, breakthrough viruses were identified in the culture supernatants with $10 \times EC_{50}$ of EMV alone, 3TC alone, ddI alone, d4T + ddI, and $5 \times EC_{50}$ of both three-drug combinations. Drug-susceptibility tests of the breakthrough viruses to each compound showed that the only viruses obtained in the presence of EMV alone and 3TC alone were significantly (215 and > 77-fold) less susceptible to EMV and 3TC, respectively.

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Long-Term Culture of HIV-1-Infected Cells with the Transcription Inhibitor K-37: Characterization of Breakthrough Viruses

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We have previously reported that 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxo-quinoline-3-carboxylic acid (K-37) is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cell cultures. Its mechanism of action studies have revealed that K-37 blocks viral transcription through the inhibition of RNA-dependent transactivators including HIV-1 Tat. To further investigate the effects of K-37 on HIV-1 replication, we have performed long-term culture of HIV-1-infected cells in the presence of K-37. When MOLT-4 and U937 cells were infected with HIV-1 (III_B strain) and cultured in the absence of K-37, p24 antigen levels in the culture supernatants reached a plateau (100-300 ng/ml) within 12 days. In the presence of K-37 (0.25 and 0.5 μM), the elevation of p24 antigen levels was delayed and could reach the same plateau level on day 16 or later. At a concentration of 1 μM, K-37 almost completely suppressed HIV-1 replication in MOLT-4 and U937 cells. However, viral breakthrough was observed in the supernatants after 1 month of the culture period, and p24 antigen levels reached a plateau after 44 days. The wild-type (III_B) and the breakthrough viruses in the presence of 0.5 μM and 1 μM K-37 (III_B/0.5 and III_B/1, respectively) were propagated once in MT-4 cells. Then MT-4 cells were infected with the same amount (10,000 $CCID_{50}$) of the propagated viruses and cultured in the absence of K-37. Interestingly, the infectivity of progeny viruses produced from the III_B/1-infected cells was found to be much lower (4 to 11-fold) than that from the III_B- or III_B/0.5-infercted cells. No significant difference was observed among the p24 levels in their culture supernatants, suggesting that the long-term culture of HIV-1-infected cells with K-37 reduces the infectivity or replication efficiency of progeny viruses.

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HIV-1 LTR- and SV40- driven gene expression is inhibited by the CMV promoter

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Because of its high-level constitutive expression, the CMV immediate early gene enhancer/promoter is frequently used in the commercially available reporter vectors encoding, e.g. β-galactosidase, luciferase or green fluorescent protein. Such constructs are used as reference plasmids which are co-transfected with gene constructs being studied for functional activity. We examined whether the CMV promoter could influence HIV-1 LTR- and SV40-driven gene expression. HEK293 and HeLa-CD4 cells were co-transfected with either the pCMV.SPORT-βgal or pCMVlacZ plasmid and the HIV-1 proviral clone HXBΔBgl, complexed with the transfection reagent Fugene. Alternatively, HEK293 and HeLa cells were co-transfected with the pCMV.SPORT-βgal and SV40 luciferase plasmids. Co-transfection of the CMV plasmids with HXBΔBgl, at a weight ratio of 4:1 reduced viral production by ~80% compared to co-transfection with the control Pol III U6 promoter. When the Pol III U6 promoter was used to transcribe the anti-HIV-1 Rev-binding aptamer [RBE(apt)], virus production was inhibited by 80% in HEK293 cells, indicating that inhibition was a consequence of Rev binding by the aptamer, and not a promoter effect. In contrast, co-transfection of the CMV-βgal and SV40 luciferase plasmids reduced luciferase activity in HEK293 and HeLa cells by ~70% and 95%, respectively, while the U6 promoted RBE(apt) plasmid had no effect. Our results suggest that titration by a strong promoter of transcription factors essential for HIV-1 replication, may not only inhibit HIV-1 production, but also artificially bias results obtained with anti-HIV-1 gene constructs.

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Electrochemiluminescent Assay for the Analysis of HIV-1 p24 Antigen from Infected Cells Using the IGEN M-series® Assay System

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In vitro HIV infection of cell lines and primary cells can release up to 50 ng/mL of p24 antigen into culture supernatants. Using currently available ultrasensitive commercial ELISA kits to analyze *in vitro* HIV production levels of this magnitude requires successive serial dilutions of samples, thus increasing intra- and inter-assay error. We have adapted the IGEN M-series® electrochemiluminescent (ECL) assay system for the detection of HIV p24 in culture supernatants. The HIV-1 p24 antigen detection assay is a 96 well microtiter sandwich immunoassay using an ECL detection endpoint based on a ruthenium labeled anti-HIV p24 monoclonal antibody. HIV containing supernatants are lysed and 200µL samples added to the wells of a 96 well microtiter plate. Twenty-five microliters (25µL) of 0.5 µg/mL ruthenylated monoclonal HIV-1 p24 antibody, and 25 µL biotinylated monoclonal HIV-1 p24 antibody pre-bound with streptavidin coated paramagnetic beads (0.5 µg/mL and 0.2 mg/mL, respectively) are then added. The reaction mixture is incubated for three hours at room temperature and detected on an IGEN M-series instrument. The p24 assay was linear with a dynamic range from 0.01-10 ng/mL. Direct comparison with the commercial Coulter p24 ELISA showed equivalent lower range sensitivity and overall quantification of supernatant p24 for both laboratory and clinical isolates of HIV-1. However, the M-series p24 assay required minimal sample dilution due to its 80-fold greater dynamic range and less sample and assay manipulation to complete the assay. The M-series p24 assay is a sensitive, broad-ranged, and efficient alternative for measuring HIV-1 p24 antigen generated by infected cells in culture.

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Treatment of T-lymphoid cells with pharmacological relevant cytarabine concentrations induces resistance to HIV-1 infection due to reduced expression of HIV-1 receptors

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Entry of human immunodeficiency virus type 1 (HIV-1) into target cells is a receptor mediated process and depends strongly on virus-receptor binding to CD4 surface antigen as well as several co-receptors such as CXCR4 and CCR5. CXCR4 is the coreceptor for T-cell line tropic virus strains whereas CCR5 is a coreceptor for macrophage-tropic strains. Dual tropic HIV-1 isolates can use either macrophage tropic or T-cell line tropic receptors. Modulation of these receptor expression on target cells may contribute to antiretroviral effects in cells. Previously we have shown that in C8166 T-lymphoid cells continuously treated with pharmacological relevant concentrations of cytarabine (Ara-C) decreased expression of CD4 and CXCR4 resulting in resistance to HIV-1 infection. This phenomenon has now been extended for several different T-lymphoid cell lines, which all showed strongly decreased CD4 and CXCR4 protein expression measured by FACS. In these cell lines a block in transcription of CD4 and CXCR4 mRNA has been demonstrated by reverse transcriptase PCR (RT-PCR) analysis of gene expression. All Ara-C resistant cell lines were completely resistant to HIV-1 infection as measured by levels of HIV-1 RNA copies and HIV-1 p24 antigen. The present results encourage further studies to show whether continuous treatment with pharmacological relevant concentrations of Ara-C may influence sensitivity of normal T lymphocytes to HIV-1 infection.

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Evaluating Reports of Adverse Effects for Anti-Retroviral Therapies: The Feasibility of Pharmacoepidemiologic Investigations in the MediCal HIV+ Population.

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A number of cohort analyses have evaluated the relationship between anti-retroviral therapy and adverse effects. Many of these cohorts have included small numbers of patients. The MediCal database, which includes the health care billing claims of all Medicaid recipients in California, provides the means studying a large cohort of HIV+ patients. We reviewed the MediCal database for all persons with a diagnosis of HIV infection between 1 January 1997 and 30 June 1999, and their associated billing claims for healthcare services provided to them. From this population, we identified all persons who received either stavudine but not AZT, or AZT but not stavudine. We identified approximately 9,200 such persons. Linkage with diagnosis codes for bone marrow suppression identified about 1,200 cases. The proportion of AZT users developing myelosuppression was 14.6 percent and for stavudine, 11.7 percent ($p<0.025$). A similar examination of lipodystrophy incidence found no difference in risk ($p>0.20$) among AZT and stavudine users . We conclude that the MediCal database is a valuable resource for identification of adverse effects of anti-retroviral therapy.

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Drug absorption studies of d4T-monophosphate prodrugs: evaluation of ester hydrolysis and transepithelial transport.

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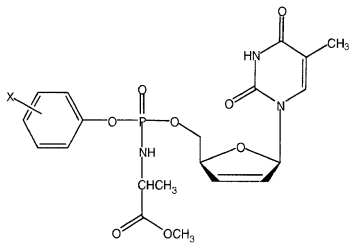
The potential for oral delivery of a representative group of d4T-MP prodrugs [1] has been examined. The permeability coefficients (apical to basolateral) of the intestinal cell line Caco-2 to the prodrugs and their stability to chemical hydrolysis were investigated, examining differences in stereochemistry at the chiral phosphate centre. In the Caco-2 monolayers the prodrugs displayed moderate to high permeability correlating with their octanol-water partition coefficients (log *P*). All the compounds showed less than 20% decomposition over a period of 20 hrs at pH 2.0, 4.6, and 7.4. Concurrent work which will be presented is addressing the extent of enzymatic metabolism in human serum and caco-2 cells, together with studies examining the role of P-glycoprotein in the transport of the prodrugs.



X = H
p-Cl
p-I
p-Me
p-OMe
p-COOMe

[1] Siddiqui, A.Q. et al. (1999) Design and synthesis of lipophilic phosphoramidate d4T-MP prodrugs expressing high potency against HIV in cell culture: structural determinants for in vitro activity and QSAR. J.Med.Chem. 42, 4122-4128.

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Antiviral and Immunomodulating Effects of the Sodium Salt of 2-chloro-5-nitrobenzoic acid on Isolated Human PBMC and HIV-Infected HuPBMC-SCID Mice.

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It seems unlikely that antiretroviral drugs alone will 'cure' HIV infections. A number of immunotherapeutic approaches are being investigated to discover the role that they may have in containing this chronic disease. These approaches include the use of therapeutic vaccines, monoclonal antibodies, cytokines and small organic molecules. In this respect, the sodium salt of 2-chloro-5-nitro benzoic acid (CNBA-Na) has been found to belong to a family of benzoic acid derivatives that induce T-cell proliferation. This costimulatory affect is most pronounced in peripheral blood mononuclear cells (PBMC) from healthy donors but less so in PBMC isolated from HIV-infected patients. Evaluation of CNBA-Na in the HuPBMC-SCID mouse model of HIV infection showed that there was an increase in the ratio of CD4+ cells compared to the CD8+ cells in all the body compartments measured. There was also a small increase in HIV replication following administration of the compound to the infected animals. Combination studies of licensed antiretroviral drugs and CNBA-Na showed there was no antagonist effects, thus any increase in HIV replication may be controlled. The mechanism of action of CNBA-Na was also investigated and *in vitro* studies indicated that its costimulatory effect is IL-2 dependent.

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Selective delivery and efficacy of Fludarabine encapsulated in red blood cells to HIV-infected macrophages

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Macrophages (M/M) infected by HIV produce large amount of virus for long period of time. It is then conceivable that the selective elimination of long-living HIV-infected M/M may be of advantage in the therapy of HIV infection. Fludarabine, a newer nucleoside analogue with documented activity in lymphoid malignancies, acts as a cytotoxic compound by inhibiting DNA synthesis after conversion to its triphosphate form (active metabolite); other mechanisms of action, as incorporation in RNA and induction of apoptosis, have also been shown. We thus evaluated, on HIV-infected M/M, the selective effect of Fludarabine encapsulated into autologous red blood cells (RBC), artificially aged to overwork the phagocytic ability of macrophages. Human primary M/M obtained from seronegative blood donors were infected with HIV-1 and then treated with free Fludarabine or Fludarabine-loaded RBC for 18 hours. Cytotoxic effect, p24 gag production and % of infected macrophages were then assessed in 4 separated experiments. Treatment of HIV-infected M/M with free Fludarabine (1uM) induced a decrease of 75% of p24 assayed 2 days after the end of treatment. Similarly, Fludarabine within RBC induced a decrease of 85.5% of p24. Cytotoxic effect of Fludarabine-loaded RBC was also evident upon HIV-infected M/M, whose number decreased about 75% compared to M/M infected but not treated. The same treatment did not affect the number of uninfected M/M. Interestingly, free Fludarabine was more cytotoxic in lymphocytes than M/M, with TC50 of 1uM and 20 uM respectively. At the same time, however, no cytotoxic effect at all could be detected in such and other non-phagocyting cells treated with Fludarabine-RBC. Finally immunofluorescence analysis shows that the treatment with Fludarabine within RBC induces a 75% decrease of p24 positive cells in HIV infected cultures of M/M In conclusion a substantial and selective antiviral and cytotoxic effect has been achieved by treating HIV-infected M/M with Fludarabine-loaded RBC. These data can provide a rationale to combine this experimental approach with other anti-HIV drug administered as free compound.

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Comparison of the Anti-HIV Activity of Racivir™ and Emtricitabine in the HuPBMC-SCID Mouse Model

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Objective: To compare the antiviral efficacy of Racivir™ (RCV, racemic FTC) and of emtricitabine [(-)-FTC, Coviracil] in the HuPBMC-SCID mouse model of HIV-1 infection.
Methods: The plasma pharmacokinetics of the orally administered compounds were first determined in uninfected SCID mice. Then, groups of nine mice were treated with either antiviral agent orally (0.01, 0.1, and 1 mg/ml) for one week after infection. The viral load in plasma was quantitated by the NucliSens assay and compared to untreated, infected mice. Viral load data were analyzed further in a pharmacodynamic model of HIV infection to compare the antiviral potency of RCV and (-)-FTC.
Results: The pharmacokinetic study in SCID mice indicated that RCV achieved a higher C_{max} and an earlier time to maximum concentration than did (-)-FTC. In HIV-infected mice, both RCV and (-)-FTC produced potent, dose-dependent antiviral activity, suppressing viral load in plasma to below detectable levels (≤ 500 copies/ml). Even though half of RCV is comprised of the plus-FTC, RCV was at least as active as (-)-FTC at the doses selected. Pharmacodynamic modeling of the viral load data indicated a marked advantage for RCV over (-)-FTC, with calculated ED_{50} values of 5.6 mg/kg/day and 15 mg/kg/day, respectively.
Conclusions: Both RCV and (-)-FTC had potent, dose-dependent antiviral activity in the HuPBMC-SCID mouse model of HIV-1 infection, and RCV was at least as potent as (-)-FTC. Pharmacokinetic studies in SCID mice provide support for the apparent advantage of RCV over (-)-FTC in this model.

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Novel Ribonucleotide Reductase Inhibitors, Didox and Trimidox, Compared to Hydroxyurea to Reverse Established Disease in the Murine AIDS (MAIDS) Model. H . Elford.[3] . C.Mayhew[1, 2], M.Inayat[1, 2], R. Sumptner[3] , V. Gallicchio[3], and[1]U. of Wolverhampton, Wolverhampton, UK; [2]U. of Kentucky, Lexington, KY; [3]Molecules For Health Inc., Richmond, VA.

Prevention of deoxynucleotide synthesis by inhibiting ribonucleotide reductase (RR) as a strategy to impair HIV replication has gained acceptance by the success of hydroxyurea (HU) to enhance the deoxynucleotide reverse transcriptase inhibitor ddI in clinical trials. However, HU as a single agent in HIV therapy, has not demonstrated significant clinical antiviral activity. On the other hand, RR inhibitors, Didox (DX) and Trimidox (TX), have shown potent antiviral activity when used alone in murine retroviral models. The antiviral activity was more pronounced with these compounds than HU especially in the HIV-infected SCID-Hu mouse model. Furthermore, these novel RR inhibitors potentiate ddI more effectively than HU. This report focuses on comparing DX, TX or HU alone to reverse established disease in the MAIDS model. MAIDS infected animals were not treated until 9 wks post infection. At this stage in this model, the infected mice have developed immunodeficiency, large viral load and profound disturbance in splenic architecture and size as well as marked hypergammaglobulinaemia. The mice were treated for 4 wks with daily injections. Since this treatment strategy targets a cellular reaction, toxicity is an issue. Therefore, the treatment effect on hematological indexes was also monitored. All 3 RRI's reduced splenomegaly below levels seen at the 9 wk time point when treatment was initiated. DX and TX were more effective than HU. Most significantly, TX and DX to a lesser extent dramatically restored splenic architecture to nearly normal levels while the HU effect was more modest but did restore the splenic architecture to a state better than the 9 wk infected observation. With regards to hematological toxicity, HU reduced peripheral blood indices, femoral cellularity, femoral CFU-GM and BFU-E progenitor cells more than TX or DX with this late stage treatment regimen. In conclusion, these data support the concept of using RRI for treatment of retrovirus infection and this approach can work in established disease. The RRI's DX and TX were more effective and less hematologically toxic than HU.

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Understanding Efficacy and Toxicity of Anti-HIV Drugs at a Molecular Level: Mechanism for the Inhibition of HIV-1 Reverse Transcriptase and Human Mitochondrial DNA Polymerase

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[1]Triangle Pharmaceuticals, Durham, NC, USA; [2]Emory University, Decatur, GA, USA; [3]Yale University, New Haven, CT, USA.

Nucleoside analog inhibitors are an essential component of AIDS therapy. The 5'-triphosphate form of these drugs inhibit viral replication by acting as alternative substrate inhibitors of the HIV-1 reverse transcriptase (RT), which results in chain-termination of viral DNA synthesis. However, long-term use of nucleoside analogs can lead to various forms of toxicity, including syndromes manifested through inhibition of the human mitochondrial DNA polymerase (Pol γ). Pol γ has both 5'→ 3' polymerase activity and 3'→ 5' exonuclease activity. The later is involved in proofreading during mitochondrial DNA synthesis, and therefore may enable mitochondria to escape the inhibitory effect of a nucleoside analog through 3'→ 5' excision of the incorporated analog. In this study, we compare the inhibitory effects of the 5'-triphosphates of a series of dideoxycytidine analogs for HIV-1 RT and Pol γ, and exonuclease cleavage of the terminally incorporated nucleotide by Pol γ. The analogs tested include the 5'-triphosphates of ddC (zalcitabine), (+)3TC, (-)3TC (lamivudine), (+)FTC, and (-)FTC (emtricitabine, Coviracil®). Using pre-steady-state kinetic analysis, each analog was studied for its maximum rate of incorporation (k_{pol}) and binding affinity for enzyme•DNA complex (K_d). Our study showed that (1) for HIV-1 RT-catalyzed RNA-dependent-DNA synthesis, the incorporation efficiency (k_{pol}/K_d) for the analogs is in the order of ddC-TP > (-)FTC-TP > (+)FTC-TP = (+)3TC-TP > (-)3TC-TP; (2) for Pol γ-catalyzed DNA synthesis, the incorporation efficiency is in the order of ddCTP > (+)FTC-TP > (+)3TC-TP > (-)3TC-TP > (-)FTC-TP; (3) for Pol γ-catalyzed excision of chain terminator, the reaction rate (k_{exo}) is in the order of (+)3TC-MP = (-)3TC-MP > (+)FTC-MP = (-)FTC-MP >> ddC-MP. In conclusion, there is clear differences between HIV-1 RT and Pol γ in terms of preferences for substrate structure. Among all of the analogs tested, (-)FTC triphosphate, a potent inhibitor of the HIV-1 RT, has the lowest inhibitory effect on Pol γ. These results are consistent with the antiviral efficacy and low mitochondrial toxicity observed in (-)FTC clinical trial studies, and support further examination of the long-term safety and efficacy of (-)FTC.

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Lack of Association Between Use of Stavudine and the Subsequent Development of HIV-Associated Lipodystrophy: A Pharmacoepidemiologic Investigation of 9,000 HIV+ Persons in the MediCal Population. David E. Lilienfeld, Bernhard Heiles, Hugh Kawabata, Jean Lian, Michael R. Stevens. Bristol-Myers Squibb Company, Princeton, NJ, USA.

The relationship between the use of stavudine and the subsequent development of lipodystrophy is controversial. Although many cohort studies of stavudine and lipodystrophy have been reported in the literature, these investigations have generally been small, with fewer than 1,000 participants. We examined this relationship in a population of HIV+ persons in the MediCal population between 1 January 1997 and 30 June 1999. MediCal provides cost re-imbursement for health care services provided to Medicaid recipients in California. We reviewed the MediCal claims database to find HIV+ persons who used either stavudine and not AZT, or AZT and not stavudine. There were 9,000 individuals in the data base meeting this criteria. We then searched the inpatient and outpatient services claims for these persons, noting for each person when a diagnosis of lipodystrophy was made. The risk of developing lipodystrophy after the use of stavudine was 6.9 percent, and after the use of AZT, 6.0 percent. This difference was not statistically significant ($p>0.20$). Proportional hazards adjustment by age, gender, race, and use of a protease inhibitor did not change this finding. Of these other factors, only age was found to be statistically associated with lipodystrophy risk; risk increased with ascending age (hazard ratio of 1.021 per year, $p<0.001$). We conclude that there is no difference in the risk of lipodystrophy among users of stavudine and users of AZT in this population, one of the largest epidemiologic studies of lipodystrophy to date.

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Adipogenic suppression induced by HIV-1 protease inhibitors is exacerbated by TNF-α and HIV-1 Tat protein. Krishna C. Agrawal, Debasis Mondal, and Vincent F. LaRussa. Dept. of Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112.

Long term use of HIV-1 protease inhibitors (PIs) has been associated with a severe lipodystrophy syndrome. We have investigated the effects of chronic PI treatment on insulin (0.1 U/ml) stimulated adipogenesis in murine 3T3-L1 cells, and in human bone marrow stromal pre-adipocytes. In addition, putative deleterious effects of a compromised adipogenic micro-environment in the presence of HIV-1 infection, were also measured by concomitant treatment of cells with either TNF-α or HIV-1 Tat protein. Effects of these treatments on insulin stimulated lipogenesis were measured in both cell models by using ^{14}C-glucose as a substrate. Lipid accumulations were quantified by measuring oil red-O staining intensity. Chronic treatment (9-12 days) of these cells with indinavir or ritonavir (0.4-50 μg/ml) produced a concentration dependent suppression of lipid accumulation (13-38%), triglyceride contents (5-49 %), and lipogenesis as monitored by glucose incorporation (21-76 %). Concomitant treatment with TNF-α (10-500 U/ml) or Tat (1-100 ng/ml) protein under these conditions exacerbated this suppressive effect induced by the PIs in a concentration dependent manner. An increase in insulin concentration to 0.5 U/ml was unable to override the suppressive effects of chronic PI treatment. However, insulin sensitization of cells via troglitazone (0.8-8 μM) treatment ameliorated this suppression as a function of its concentration. Since insulin resistance and visceral obesity has been linked to changes in levels of fibrinolytic proteases, e.g. plasminogen activators (PAs) and their inhibitors (PAIs), we have also monitored the expression of these endogenous proteases in insulin stimulated cells by using a chromogenic assay for plasmin. The PIs increased the plasmin production and decreased the PAI activity in 3T3-L1 cells at 48 hr after stimulation with insulin in contrast to undifferentiated adipogenic cells in which the PIs produced no significant effect. These data suggest that the pre-adipocytes in HIV-1 positive patients may be predisposed to the toxic effects of PIs in the presence of TNF-α and HIV-1 Tat protein, and therefore manifest an exacerbated lipodystrophy. Furthermore, these data also suggest that the HIV-1 PIs may cross-inhibit the function of certain host proteases and may thus disrupt the process of adipogenesis.

Oral Session III: Herpesvirus Infections

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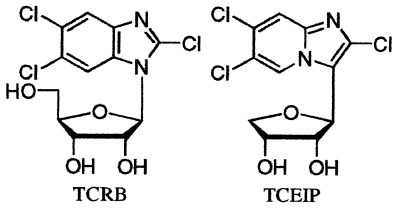
Design and Synthesis of 2,6,7-Trichloro-3-(erythrofuranosyl)-imidazo[1,2-*a*]pyridines as Selective Inhibitors of HCMV Replication. J.D. Williams, K.S. Gudmundsson, J.C. Drach, and L.B. Townsend, Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA.

The benzimidazole nucleoside analog TCRB is a potent and selective inhibitor of HCMV replication *in vitro*, but is rapidly degraded *in vivo*, limiting its potential as a therapeutic agent. C-Glycosides are not susceptible to glycosidic bond hydrolysis, and thus should be effective in surviving the hydrolytic degradation observed with TCRB. Toward this end, the four isomers of 2,6,7-trichloro-3-(erythrofuranosyl)-imidazo[1,2-*a*]pyridine (TCEIP) have been synthesized, using Heck coupling methodology to establish the glycosidic bond. The resulting dideoxy, didehydro erythrofuranosyl intermediate was produced as a racemic mixture which was dihydroxylated with either AD mix-α or AD mix-β to produce a mixture of anomers of either the D or L erythrofuranosyl compounds. After purification, some of the compounds demonstrated good activity against HCMV, but initial experiments suggest that the mode of action is different from that of TCRB. These studies have been supported by NIAID grant U19-AI31718 and NIH grant T32-GM07767.



TCRB TCEIP

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Unsymmetrical N-Alkyl Indolocarbazoles: Potent Inhibitors of Human Cytomegalovirus Replication.
M.J. Slater, S. Cockerill, R. Baxter, R.W. Bonser, K. Gohil, E. Robinson, N. Parry, R. Randall and W. Snowden. Glaxo Wellcome, Gunnels Wood Road, Stevenage, SG1 2NY, UK

In our search for novel, safer treatments of Human cytomegalovirus (HCMV) disease, we discovered and recently reported that the indolocarbazole Arcyriaflavin A is a potent and selective inhibitor of HCMV replication (*Bioorg. Med. Chem.* (1999) 7, 1067). In order to optimise the antiviral activity and explore structure-activity relationships in this series, we synthesised a range of unsymmetrical N-alkyl substituted indolocarbazoles. The synthetic routes employed, and the biological profiles of these targets in vitro are described. This work has identified 12-ethyl-12,13-dihydro-5H-indolo[2,3a]pyrrolo[3,4c]carbazole-5,7(6H)-dione as one of the most potent inhibitors of HCMV replication in cell culture reported to date (IC_{50}=0.019μM).

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Inhibition of Human Cytomegalovirus Replication by 2-Hydroxymethylcyclopropylidenemethyl Purines Involves Viral Gene Products. J.C. Drach, J.M. Breitenbach, and K.Z. Borysko, University of Michigan, Ann Arbor, Michigan 48109; Y.-L. Qiu and J. Zemlicka, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201, U.S.A.

We have previously described a series of novel hydroxymethylcyclopropylidenemethyl purines that have broad-spectrum antiviral activity (Qiu *et al., J. Med. Chem.* **41**:10-23, 1998; Qiu *et al., Antiviral Chem. Chemother.* **11**:191-202, 2000). The racemic Z-isomers of adenine (Z-Ade), guanine (Z-Gua), and 2-amino-6-cyclopropylamine (Z-ACPAP) analogs were particularly active against human cytomegalovirus (HCMV); IC_{50}'s ≅ 0.5-5-2 μM in plaque assays and IC_{90}'s ≅ 1-2 μM in yield assays. HCMV resistant to ganciclovir (GCV) due to a mutation in UL97 (L595S) was slightly resistant to Z-Ade but not to Z-Gua nor Z-ACPAP. HCMV resistant to GCV due to a mutation in DNA polymerase was sensitive to both Z-Ade and Z-Gua. *In vitro* metabolism experiments with HCMV-infected cells suggested phosphorylation of [^{3}H]Z-Gua and [^{3}H] Z-ACPAP to a limited extent. Both Z-Ade and Z-Gua were used to select drug-resistant HCMV. Incubation in the presence of step-wise increasing concentrations of each compound was continued until cytopathology was apparent in most cells. Supernatants were used to infect new cultures of HFF cells until virus would not grow or until drug cytotoxicity interfered (64 and 32 μM, respectively). Virus isolated by three plaque purifications in the presence of compounds and by two limiting dilutions in their absence was approximately 10-fold resistant in plaque reduction assays to the compound used for the selection. The isolates also were resistant to the other two purine analogs and to GCV. A mutation (C603T) was detected in UL97 of the strain selected for resistance to Z-Gua but not in the strain selected for resistance to Z-Ade. We conclude that at least one viral gene product is involved in the activity of these compounds. Additional phenotypic and genotypic characterizations of the drug-resistant strains are in progress. This study was supported by grants U19-AI31718, P01-AI46390 and R01-CA32779 from N.I.H.

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The Effect of Benzimidazole Nucleosides on Human Cytomegalovirus Replication in the SCID-hu Retinal and Thymus/Liver Tissue Implant Models. D.J. Bidanset[1], R.J. Rybak[1], D.J. Collins[1], C.B. Hartline[1], J.C Drach[2], L.B. Townsend[2], K.K. Biron[3] and E.R. Kern[1]. [1]The Univ. of Alabama Sch. Of Med., Birmingham, AL, [2]Univ. of Michigan, Ann Arbor, MI and Glaxo Wellcome Inc., Research Triangle Park, NC.

Human cytomegalovirus (HCMV) is a ubiquitously distributed β-herpesvirus that can cause a wide variety of clinical manifestations in the immunocompromised host. As HCMV does not infect or replicate in non-human cells and tissues, there are few animal models currently available to evaluate antiviral therapies for these infections. In the current studies, we utilized two different models in which severe combined immunodeficient (SCID) mice were implanted with human fetal tissue that was subsequently infected with HCMV. In one model, human fetal retina was implanted into the anterior chamber of the SCID mouse eye and inoculated 8-12 weeks later with 5000-10,000 pfu of HCMV. In the second model, human fetal thymus and liver (thy/liv) was implanted under the kidney capsule of the SCID mouse and inoculated 16-20 weeks later with 5000-10,000 pfu of HCMV. At various times after infection, implanted tissues were removed, homogenized and HCMV titers quantified by plaque assay. Previous experiments had indicated that in both models, the replication of the Toledo strain of HCMV increased through 21-28 days and then gradually decreased to undetectable levels by 8 weeks post infection. To determine the efficacy of three benzimidazole nucleosides, 2-bromo-5, 6-dichloro-1-β-D-ribofuranosyl benzimidazole (BDCRB), GW275175X (175X), and GW257406X (1263W94), treatment was initiated 24 hr after infection and continued for 28 days. Treatment consisted of either placebo, 25 mg ganciclovir (GCV)/kg administered i.p. twice daily, 33 or 100 mg BDCRB/kg administered i.p. twice daily, or 75 mg 1263W94 or 175X administered orally twice daily. The results in the retinal tissue model indicated that 1263W94 and BDCRB were effective in reducing HCMV replication about 4-fold through 21 days post infection when compared with the vehicle control. In the thy/liv tissue model, all three benzimidazole nucleosides were effective in inhibiting HCMV replication. By 28 days after infection, HCMV replication was inhibited by approximately 30-3000 fold in comparison to the vehicle control. GCV was effective in both models inhibiting HCMV infection by 5-3000 fold. These data indicate that the benzimidazole nucleosides were efficacious in these two animal models and should be active against the various HCMV infections that occur in the immunocompromised host.

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Broad Spectrum Anti-herpesvirus Activity of Novel 4-Oxo-Dihydroquinolines Which Target the Viral Polymerase.

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We have identified novel non-nucleoside herpesvirus polymerase inhibitors belonging to the 4-oxo-dihydro-quinoline (4-oxo-DHQ) class which selectively inhibited the viral polymerases of HCMV, HSV, EBV, VZV, and HHV-8 but did not inhibit human α, δ, or γ polymerases. In cell culture, using either plaque reduction or virus yield reduction assays we show that the 4-oxo-DHQ's inhibit human and rodent cytomegaloviruses (ED_{50}, 0.3-5 μm) and are active against a panel of clinical isolates of human CMV. In addition, these compounds have potent activity against varicella zoster virus (ED_{50}, 0.2-3 μm), against simian varicella virus, and are active against a panel of clinical isolates of VZV. Laboratory isolates of HSV-1 and HSV-2 were also inhibited in cell culture by this class of compounds (ED_{50}, 4-8 μm). In addition, we have shown that laboratory derived strains of ganciclovir and cidofovir resistant cytomegalovirus and acyclovir resistant strains of herpes simplex virus remained sensitive to the antiviral inhibition of the 4-oxo-DHQ's. The 4-oxo-DHQ's were inactive against several non-herpesvirus DNA and RNA virus families (ED_{50}, >100 μm). PNU-183792, one of the lead compounds in this class, was orally bioavailable and exhibited moderate rates of clearance in rats. PNU-183792 exhibited potent antiviral activity against MCMV in BALB/c mice when administered by the oral route.

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Selective Abolishment of the Pyrimidine Nucleoside Kinase Activity of Herpes Simplex Virus Type 1 Thymidine Kinase by the Ala167Tyr Mutation

J. Balzarini,[1] B. Degrève,[1] S. Liekens,[1] R. Esnouf [2] & E. De Clercq[1]

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Models for 167-mutated HSV-1 thymidine kinase (TK) produced by MidasPlus and optimised using RotSearch showed that a tyrosine mutation at amino acid position 167 of HSV-1 TK may allow effective discrimination between thymidine (dThd) and ganciclovir. Therefore, we have constructed the Ala167Tyr mutant HSV-1 TK. In contrast with wild-type HSV-1 TK the mutant Ala167Tyr TK lost the pyrimidine nucleoside kinase activity while retaining significant purine nucleoside kinase activity. Indeed, compared with wild-type HSV-1 TK, the purified HSV-1 Ala167Tyr TK mutant enzyme was heavily compromised in phosphorylating (*E*)-5-(2-bromovinyl)-2'-deoxyuridine and dThd, whereas its ability to phosphorylate ganciclovir and lobucavir was only slightly (2-fold) affected. In addition, a markedly decreased competition of thymidine with GCV phosphorylation by the mutant Ala167Tyr TK was observed, suggesting that dThd was poorly recognised as substrate for the mutant enzyme. These findings add more insights in the molecular interaction of pyrimidine and purine nucleosides with HSV TKs. They may also have interesting implications for HSV-1 TK-based gene/chemotherapy of cancer by eliminating potential endogenous competition of ganciclovir with the natural substrate dThd, which may, in turn, result in an increased cytostatic potential of the purine nucleoside analogues.

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Acyclovir Cream for the Treatment of Herpes Simplex Labialis: The Results of Two Double-Blind, Placebo-Controlled Trials. SL Spruance, J Johnson, T Spaulding and the ACV Cream Study Group. U of Utah, Salt Lake City, UT; and GlaxoWellcome, Research Triangle Park, NC, USA.

Acyclovir (ACV) cream has been widely available for the treatment of herpes labialis in countries outside the United States for over a decade. To date, there are only a few small clinical trials describing its efficacy. We conducted two randomized, double-blind, placebo-controlled, multicenter clinical trials comparing ACV cream and a cream vehicle control. The study subjects were adults in good health other than recurrent herpes labialis. Patients were screened for eligibility, randomized and given study medication, and told to self-initiate topical treatment 5x/day for 4 days beginning within one hour of the onset of the next episode. The number of patients who treated a lesion was 686 in Study 1 and 699 in Study 2. The primary efficacy variable was episode duration, defined as the time from the initiation of treatment to the cessation of lesion signs and symptoms. In Study 1, the mean duration of recurrent episodes was 4.3 days for patients treated with ACV cream and 4.8 days for those treated with the vehicle control (HR=1.23, 95% CI: 1.06-1.44, p=0.007). In Study 2, the mean duration of recurrent episodes was 4.6 days for patients treated with ACV cream and 5.2 days for those treated with the vehicle control (HR=1.24, 95% CI: 1.06-1.44, p=0.006). Efficacy was apparent both when therapy was initiated "early" (prodrome or erythema lesion stage) and when initiated "late" (papule or vesicle stage). There was a statistically significant reduction in the duration of lesion pain in both studies. Adverse events were mild and infrequent in both treatment groups.

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Two-Day Treatment with Acyclovir Decreases Duration of Lesions and Viral Shedding in Genital HSV-2 Infection.

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Standard therapy for recurrent genital herpes involves multiple doses of antiviral medication for 5 days. To investigate the effect of high-dose, short-duration therapy for recurrent genital HSV-2 infection, we conducted a double-blind, placebo-controlled trial of acyclovir 800 mg po tid for 2 days. Participants self-initiated study drug at prodrome or first notice of lesions, and were evaluated in clinic within 24 hours. Patients were seen in clinic for the first 7 days of lesions, then every other day until healing. Swabs for viral cultures were obtained daily until healing. Each participant could receive the same therapy (acyclovir or placebo) for up to two subsequent recurrences. 134 subjects with a history of genital herpes and serologically confirmed HSV-2 infection were enrolled into the study. Of these, 56 (42%) were observed through two recurrences and 28 (21%) were observed through one recurrence. 62% of participants were women and 38% were men. In an intent-to-treat analysis, the duration of lesions was shorter in patients receiving acyclovir 800 mg po tid for 2 days compared with placebo, median 3.5 vs. 6.0 days, (P=0.001, Mann-Whitney test). The proportion of patients with at least one viral isolate during a recurrence was 58% among acyclovir recipients vs. 91% among placebo recipients, (P=0.002, chi-square). The proportion of aborted episodes was also higher among the acyclovir recipients compared with placebo recipients, although not significantly, 15% vs. 7%, respectively, (P=0.18, chi-square). A 2-day regimen of high-dose acyclovir shortens the duration of genital herpes episodes and viral shedding, and may be preferred by some patients to 5-day regimens. Such short regimens for recurrent genital herpes should be investigated with new antiviral agents.

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***cyclo*Sal-BVDUMP triesters - Synthesis, properties and anti-EBV activity of new potential pronucleotides**

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5-[(*E*)-2-Bromovinyl]-2'-deoxyuridine (BVDU) is a potent and highly selective inhibitor of the replication of several herpes viruses. The selectivity as inhibitor primarily depends upon a specific activation by the virus encoded thymidine kinase (TK) to the mono- and diphosphate and finally by cellular enzymes to the triphosphate. As its triphosphate BVDU can act either as an inhibitor of the cellular DNA polymerase or alternate substrate which when incorporated in DNA would render the DNA more prone to degradation. Some limitations for the use of BVDU are known. There is a lack of activity during virus latency because of missing viral TK. Drug resistant virus strains are known. Additionally, BVDU will be degraded from the bloodstream by pyrimidine nucleoside phosphatase within 2–3 hours to the free pyrimidine nucleobase BVU. A new series of lipophilic *cyclo*Sal-BVDUMP derivatives will be presented as potential prodrugs of the antiviral agent BVDU. To these compounds the 5'-*cyclo*Sal-masking group technique has been applied in order to achieve an increase in membrane penetration and the intracellular delivery of BVDUMP. Beside the lead derivative bearing a free 3'-hydroxy group, this position has been modified by different 3'-*O*-ester of aliphatic carboxylic esters and α-aminoacids having natural and non-natural C-α-configuration for structure/activity relationship studies. It was proven that BVDUMP was liberated by means of chemical hydroysis. In addition to the synthetic access to these compounds different physicochemical properties of the new derivatives were studied and will be presented, i.e. lipophilicity and hydrolysis behavior in different media. All compounds have been evaluated for their anti-EBV activity. A few compounds exhibited promising antiviral activity while BVDU was entirely inactive. This is the first example of a successful delivery of a 3'-OH bearing nucleoside analogue from a pronucleotide system reported so far.

Oral Session IV: Minisymposium – No Abstracts

Poster Session II: Retrovirus II, Herpesvirus, Poxvirus Infections

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***In Vitro* Strategies for the Preclinical Development of Topical Microbicides: Classes of Potential Inhibitors**

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One of the current challenges in anti-HIV drug discovery is the inhibition of virus transmission between sexual partners. We have developed a series of microtiter-based, high-throughput assays to evaluate the ability of anti-HIV compounds to be used as topical microbicides, including both CD4-independent (ME180) and dependent (GHOST X4/R5) cell-to-cell *in vitro* virus transmission assays. We have identified a variety of classes of active inhibitors, including polyanionic molecules, surfactants, natural products, peptides, proteins, heterocycles, virucidal agents and other anti-HIV agents. Efficacy and toxicity (especially against common flora of the vagina, such as Lactobacillus) of candidate compounds is determined in conditions that mimic the type of environment in which the compound will be required to work, including the effects of pH and mucopolysaccharides, as well as other conditions involving time of infection, treatment schedule and multiplicity of infection. An integral part of the assessment of any topical microbicide candidate is demonstration of appropriate range and mechanism of action compatible with a topical microbicide. Range of action assays evaluate the ability of candidate compounds to act against a variety of wild-type, drug-resistant, laboratory-derived and clinical strains of virus, including HIV-2 and SIV. Range of action assays also include supplementary studies for activity against bacterial and fungal pathogens. Mechanism of action assays, encompassing both biochemical/enzymatic and cell-based assays, are employed to further define the activity of the compound in intact cells. For topical microbicide inhibitor candidates, we routinely evaluate the ability of these compounds to inhibit virus-cell attachment (CD4-gp120 interaction) and cell-cell fusion. We have also designed assays which evaluate the ability of compounds to inhibit virus entry after formation of the attachment/fusion complex (virus gp120 and gp41 interaction with cell surface CD4 and chemokine coreceptors). Assays are performed to determine the relative ease of selecting for drug resistant virus strains in culture and to define the interactions of the compounds when used in combination with other active agents. Finally, candidates can be assessed in non-human primate models for *in vivo* efficacy following vaginal or rectal challenge, using RT and Env SHIV viruses.

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Development of a pH Transitions Assay For Evaluation of Topical Microbicides Candidates.

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The identification of new topical microbicides to prevent sexual transmission of HIV-1 is a priority for controlling the spread of AIDS in developed and under-developed countries. However current topical screening approaches lack the ability to mimic crucial events that occur upon initial exposure to HIV-1. It is well documented that coitus results in a transition of normal vaginal pH from 4.0 to 4.5 to around pH 7.0. Effective topical microbicides must display stability within this pH range, but current topical microbicide screening programs either do not assess efficacy at low pH or the effect of the pH transition on virus transmission and compound efficacy. Preliminary studies have shown that treatment of a number of representative low passage subtype viruses results in dramatic reductions in virus infectivity below pH 5.0. However H9 cells chronically infected with the clinical SK-1 strain of HIV-1 maintained the ability to transmit virus following exposure to pH 4.5. In order to more closely mimic the *in vivo* transmission situation we adapted the cervical epithelia cell line ME180 to survive at pH 4.5 for 4 h. H9/SK-1 cells are added in the presence of the candidate topical microbicide and the pH transitioned to 7.0 by addition of a buffering solution, and cell to cell transmission assessed by p24 production following a 4 H pH 7.0 transmission interval. Virus was successfully transmitted and inhibited by a number of potential topical microbicide candidates in this system. We have developed a pH transition assay to mimic the transition in vaginal pH that occurs with coitus. The application of this assay to topical microbicide screens should increase the success of identifying successful topical microbicide candidates.

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DABOs as candidates to prevent sexual transmission of HIV.

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Heterosexual transmission of HIV-1 is the major cause of the ongoing AIDS epidemic. The absence of a HIV vaccine, the lack of access to effective anti-HIV therapy in Third World countries, and the persistence of high viral loads in endocervical fluid and semen even in patients with treatment-suppressed HIV in their blood, has placed new emphasis on the development of topical agents capable of reducing sexual transmission of HIV.
Detergent-based vaginal microbicides currently in use, in addition to their high contraceptive failure rates, cause mucosal erosion and local inflammation that might increase the risk of heterosexual HIV transmission. Hence, there is a need for new compounds without detergent-type action. A variety of strategies have been proposed and are being investigated: (i) reconstitution of the acidic vaginal barrier (buffers or estrogen); (ii) enrichment of the vaginal flora with lactobacilli genetically engineered to produce a protein capable of preventing HIV from binding to cell surfaces; (iii) topical use of cell-fusion blockers (from sulfated polysaccarides to cyanovirin) or monoclonal antibodies that bind to HIV envelope proteins.

Recently, it has been reported that the NNRTI 5- isopropyl-2-((methylthiomethyl)thio)-6-benzyl)-pyrimidin-4-one(1H)-one (MeSMe-DABO), possesses spermicidal activity unrelated to cytotoxicity. Since our group has been involved in the development of NNRTIs (and a variety of DABOs in particular), we deemed it interesting to investigate the dual anti-HIV and spermicidal activity of the whole class of DABOs. Data will be presented on the DABOs clinical potential as topical agents to prevent sexual transmission of HIV.

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Development of a cell line with altered sensitivity to the vaginal microbicide C31G: Investigating a surfactant's mechanism of action. B.J. Catalone[a], S.R. Miller[a], F.C. Krebs[a], D. Malamud[b], and B. Wigdahl[a]. [a]Department of Microbiology and Immunology, Penn State College of Medicine, Hershey, PA, USA and [b]Department of Biochemistry, University of Pennsylvania School of Dental Medicine, and Biosyn, Inc., Philadelphia, PA, USA

Current preventative strategies to control the HIV/AIDS epidemic have been unsuccessful. A proposed strategy is the development of a female-controlled vaginal microbicide which would be effective against a broad-spectrum of sexually transmitted pathogens, including human immunodeficiency virus type 1 (HIV-1). One class of broad-spectrum agents consists of surfactants, or surface active compounds which prevent transmission by killing or inactivating the pathogenic agent. Although surfactants are known to disrupt phospholipid membranes, the specific mechanism of action is unknown. Analysis of cytotoxicity profiles has indicated that three surfactants, non-oxynol-9 (N-9), sodium dodecyl sulfate (SDS), and C31G, each possess distinct mechanisms of action. To investigate the mechanism of action of these surfactants, we developed human cervical epithelial cell lines (HeLa) treated with each surfactant for an extended period of time and then determined if these cells exhibited altered sensitivity or resistance to the treatment agent. Prolonged treatment of HeLa cells with C31G, but not N-9 or SDS, resulted in an increase in cell survival when challenged with the treatment drug. C31G-treated cells were also less sensitive to N-9, while cellular sensitivity to SDS was unchanged. In addition, altered cell populations returned to pre-treatment sensitivities in a time-dependent manner following removal of C31G treatment, indicating that a metabolic, rather than genetic, change was likely to be responsible for the observed increase in cell survival. Future studies will focus on characterizing lipid and protein differences in untreated versus C31G-treated cells, and identifying differentially expressed genes between these two cell populations using microarray analysis.

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Anti-HIV Components of OTC Vaginal Preparations. S. Baron, J. Poast, H. Lee, M. Cloyd, and D. Nguyen. Department of Microbiology & Immunology, University of Texas Medical Branch, Galveston, TX, USA

Some commercial over-the-counter (OTC) sexual preparations such as Astroglide, KY Liquid, Vagisil, and ViAmor inhibit HIV or infected leukocytes. These preparations have the advantages of safety, availability, low cost, convenience and choice by men or women. Our objective was to determine the active components of the lubricants to understand the inhibitory mechanisms and to develop improved preventives. We determined the *in vitro* inhibition of HIV-infected human leukocytes and free HIV after treatment with various OTC lubricants in comparison with their components. We also determined the time for action, titer, and activity against four strains of HIV and activity on HIV-infected human lymphocytes (CEM) and human monocytes (THP-1). Components of the OTC lubricants are active against HIV-infected cells and free HIV. These components were active in a dilution range of 1:2 to 1:12 against infected cells and free virus. They were able to disrupt the infected leukocytes within less than 5 minutes and thereby inhibited HIV production greater than 25 to 100-fold. The active OTC preparations contain such components. The stability at 37°C is under study. Thus, active components of the OTC lubricants that inhibit HIV are being identified and characterized. These OTC lubricants and their active components should be considered for clinical trials because of their FDA approval for safety and their wide availability, low cost, convenience, and choice by either men or women.

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Effectiveness of 3-Methyl-*cyclo*Saligenylnucleotides (3-Me-*cyclo*Sal-NMP) of acyclovir, ganciclovir and brivudine against thymidine kinase (TK) positive and TK-deficient HSV-1 strains

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TK-deficient HSV-1 strains, which are frequently observed among immunocompromised patients, are unable to convert nucleoside analogs into the corresponding monophosphates thus withstanding the usual HSV therapy with nucleoside analogs. The *cyclo*Sal pronucleotide concept was successfully applied to bypass the HSV-1-TK-dependent phosphorylation step of acyclovir (ACV) by the controlled release of ACVMP from the membrane-permeable lipophilic prodrug *cyclo*Sal-ACVMP [Meier et al. (1998) Antiviral Chem. Chemother. 9, 389-402]. In a comparative study, we examined the 3-Me-*cyclo*Sal-NMPs of acyclovir (ACV), ganciclovir (GCV) and brivudine (BVDU) for their antiviral and cytotoxic effects in HSV-1- and HSV-1-TK$^-$-infected Vero cells. The rank order of antiviral activity deduced from the IC_{50} values (µg/ml) was found to be as follows: in the TK$^+$ strain of HSV-1, *cyclo*Sal-ACVMP (0.2) > BVDUMP (0.7) > GCVMP (3.3), in the TK$^-$ strain of HSV-1, *cyclo*Sal-ACVMP (0.2) > GCVMP (3.1) > BVDUMP (~180). This result reveals that *cyclo*Sal-ACVMP is the most favorable TK-independent anti-HSV-1 pronucleotide of the *cyclo*Sal family. In contrast, 3-Me-*cyclo*Sal-BVDUMP, although very effective against HSV-1, failed to inhibit the TK$^-$ strain. Obviously, 3-Me-*cyclo*Sal-BVDUMP penetrates into the cell, hydrolyzes to liberate BVDUMP but will not be further metabolized to the active BVDUTP. This is most probably due to the lack of viral thymidine kinase, whose thymidylate kinase function is necessary to phosphorylate BVDUMP to BVDUDP.

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The Dendrimer BRI-2999 Inhibits Both Virus Entry and Late Stages of Herpes Simplex Virus Replication. Y. Gong[1], D. Cheung[1], A. Wen[1], D. Leung[1], B. Matthews[2], G. Holan[2], J. Raff[2], S.L. Sacks[1]. 1. Viridae Clinical Sciences Inc., 1134 Burrard Street, Vancouver, British Columbia, V6Z 1Y8, Canada, 2. Starpharma Limited, 343 Royal Parade, Parkville, Victoria 3052, Australia.

Dendrimers are macromolecules with broad-spectrum antiviral activity and minimal toxicity effective in animal models in preventing transmission of herpes simplex viruses (HSV) infection. In order to further understand the mechanism of action, and toxicity profiles of the dendrimer BRI-2999 against HSV, we investigated *in vitro* activities as follows: modified plaque reduction assays for BRI-2999 showed that 50% effective concentrations (EC_{50}) determined by pre-treatment of cells with BRI-2999 were 30.1 nM for HSV-2 and 60.2 nM for HSV-1, respectively. BRI-2999 was not toxic to either Vero or Hela-229 cells at concentrations up to the highest tested (CC_{50} greater than 60.2 μM). BRI-2999 abolished HSV-2 adsorption to Vero cells at concentrations of 180 nM. Additionally, BRI-2999 was shown to be effective on cells already infected with HSV with $EC_{90\text{-}100}$ approximately 1.8 μM for HSV-2 and 3.6 μM for HSV-1. Results from Southern blot hybridization indicated that BRI-2999 inhibited DNA synthesis in HSV infected cells, and pre-treated cells. We conclude that BRI-2999 inhibits HSV entry into susceptible cells and also DNA replication. Our data indicate that BRI-2999 is a potent inhibitor of HSV-1 and –2. Further studies are indicated to elucidate details of these dual sites of action.

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Synthesis and antiviral activity of novel *C*-methyl-substituted cyclopropyl nucleosides

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Recently, new classes of nucleoside analogues framed of core cyclopropyl backbone have been reported to have potent antiviral activity. Among them, Synadenol has a potent antiviral action against HCMV and another 9-[[cis-1',2'-bis(hydroxymethyl)cycloprop-1'-yl]methyl-guanine (A-5021) has an extremely potent antiviral activity against HSV-1. Based on these considerations, we became interested in the biological activities of *C*-carbon-substituted acyclic nucleosides and synthesized a series of methyl-*C*-branched-chain cyclopropyl nucleosides to clarify the structure-activity relationships. To synthesize the targeted nucleosides, D-isopropylidene glyceradehyde **2**, obtained from 1,2:5,6-di-D-isopropylidene D-mannitol **1** was reacted with triethyl 2-phosphonopropionate by Wittig reaction to give (*E*)-α,β-unsaturated ethyl ester **3**. The ester **3** was reduced to the alcohol **4**, which was reacted with TBDPSCl to protect hydroxy group and followed by Simmons-Smith type reaction to give the desired cyclopropyl derivative **6** in 83% high yield. The cyclopropyl intermediate **6** was deprotected to give the free alcohol **7**, which was activated by tosylation and condensed with some purine bases (adenine, 2-amino-6-chloropurine et al.) by S_N2 reaction. The isopropylidene group of **9-11** was removed by 80% CH_3COOH to give the diol nucleosides (**12-14**), which was reacted with $NaIO_4$ and followed by the reduction to give the desired nucleosides **15-17** in high yield. The antiviral activities of the nucleosides against HSV-1, HSV-2, HIV-1, HCMV and HBV were examined *in vitro*. Unlike the hydroxy methyl-*C* substituted guanine (A-5021), only the adenine nucleoside **15** is moderate active against HIV-1 without showing significant toxicity to the host cell. These results may be ascribed to the conformational change of the sugar-like cyclopropyl moiety and nucleobase.

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Inhibitory Effect of the Seaweed Polysaccharide against Herpes Simplex Viruses

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HSV-1 and HSV-2 are enveloped viruses which are the causative agents of many common infections and life-threatening diseases. So the search for selective antiviral agents has been vigorous in recent years because there are side effects of long term treatment with Aciclovir (ACV), the current antiherpetic drug, and occurrence of ACV resistant mutants. In our study, inhibitory effect of the polysaccharide from *S. patens* in Hong Kong against Herpes simplex viruses was investigated. The antiviral activities were evaluated by the cytopathic effect (CPE) inhibition assay and plaque reduction assay. This antiviral substance has been proved to be polysaccharide with M.W. about 670kDa. The 50% plaque reduction dose of the polysaccharide for HSV-1 and HSV-2 was 6.26μg/ml, 3.125μg/ml respectively in Vero cells. Pre-treatment of Vero cells with the polysaccharide did not protect cells from infection by the virus. By contrast, preincubation of the virus with the polysaccharide resulted in a concentration-dependent reduction of remaining infectivity. 90-98% inhibition was attained if the compound was present either only during virus penetration or during the whole period of the plaque assay. However when the polysaccharide was only present after virus penetration, it was no longer effective. If the polysaccharide was present after virus adsorption, it also gave a lower reduction in plaque formation as compared to the control. The results sugest that the polysaccharide may inhibite HSV by effecting the virus-cell binding and virus-cell fusion. The polysaccharide did not exhibit cytotoxicity up to 2mg/ml in Vero cells and 900μg/ml in Hep-2 cells by tetrazolium salt method. The potent nontoxic extract from *S. patens* is the promising source of natural antiviral agent for treating herpes virus infections.

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Hydroxytolan Analog Inhibition of HSV-1 Replication

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Resveratrol, a polyphenol, was shown to have antiherpetic activity (Antiviral Res., 43: 145-155, 1999). We synthesized several analogs of this polyphenol, and tested them for activity against herpes simplex virus type-1 (HSV-1). Polyphenol cytotoxicity was measured by using a standard MTT colorimetric assay. The toxicity was reported as the cytotoxic dose (CD_{50}) in μM, based on a 50% reduction of in the viability of Vero cells. Studies on inhibition of viral replication were performed in Vero cells by a standard plaque assay. One of the compounds, a dihydroxytolan, was found to have significant anti-HSV activity. Viral replication studies have shown that this compound inhibited HSV-1 replication in a dose dependent and reversible manner. Reduction in HSV-1 yield by the dihydroxytolan was not through direct viral inactivation or by inhibition of virus attachment to the cell. The compound seems to target an essential element in the immediate-early phase of virus replication. Hydroxytolan analogs differ structurally from the nucleoside analogs currently used to treat HSV infections and represent a new class of antiherpetic compounds.

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Studies on Antiviral Antibiotic 16704A, P.Z.Tao, S.Y.Si, X.B.Sun, Institute of Medicinal Biotechnology, CAMS, Beijing 100050, P.R.China

Screening for antiviral substances from the secondary metabolites of microorganisms is one of important resources for the searching leading antiviral compounds. A strain of streptomyces isolated from a soil sample collected in Qu-Jing area of Yunnan province of China displayed a broad spectrum antiviral activities in our routine screening program. The antibiotic 16704A was isolated by means of X-5 macro-porous resin column chromatography, extraction with organic solvents and RP-8 medium pressure column chromatography. Based on physico-chemical properties and spectral data of FAB-MS, EI-MS, UV and ^{1}H-NMR of 16704A, it was identified as bafilomycin A1 which was found as insecticide antibiotics with a structure of 16-membered macrolide before. In vero cells 16704A showed anti-herpes simplex virus type 1, type 2 and vesicular stomatitis virus activities with IC50s of 0.65, 0.78 and 0.39 μ g/ml respectively by CPE method. The selective indexes were 14.7, 12.2 and 24.4 respectively. The IC50s by PFU method were 0.36, 0.47 and 0.11 μ g/ml respectively each with selective index of 26.5, 20.3 and 86.6. As we know the antiviral activities of bafilomycin A1 was not found in the literatures.

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Effect of Two Novel Thymidine Phosphorylase Inhibitors on the Degradation and Antiviral Efficacy of (*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU)

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Thymidine phosphorylase (TPase, EC 2.4.2.4) has proved to be responsible for the degradation of 2'-deoxyuridine derivatives, including the well-established antiherpetic agent (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU). Based on the available X-ray crystal structure of *E.coli* TPase, we have designed two novel inhibitors of this enzyme: 7-deazaxanthine (7DX) and a multisubstrate analogue inhibitor 7DX-$(CH_2)_8$-P(O)$(OH)_2$. Extensive kinetic experiments with both compounds have shown differences in their inhibitory behavior: 7DX competed with thymine in a competitive/mixed fashion and with inorganic phosphate (Pi) in a non-competitive fashion, whereas 7DX-$(CH_2)_8$-P(O)$(OH)_2$ proved competitive/mixed against both thymine and Pi. Combination experiments of these inhibitors with BVDU revealed that degradation of BVDU by TPase could be significantly suppressed in the presence of the novel inhibitors (i.e. 90% inhibition of BVDU degradation in the presence of 100 μM 7DX). We now wanted to establish whether the inhibitors are able to prevent BVDU degradation in cell culture and whether combination with BVDU in cell culture is advantageous in potentiating the antiviral activity of BVDU against wild-type and thymidine kinase-deficient herpesvirus strains. Preliminary data point to a potentiating effect of the activity of BVDU against TK-deficient HSV-1 and VZV strains by 7DX.

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Sensitive, Automated, Non-Radioactive Detection of Herpes Simplex Virus DNA in Microtiter Plates. D. W. Selleseth, C.L. Talarico, M.G. Davis, R.J. Harvey, and R.W. Jansen, Glaxo Wellcome, Inc. RTP, NC

Cell-based compound screens that utilize viral nucleic acids as targets are often hampered by tedious, non-automated methods required for detection of low-concentration targets. Unlike dot-blot formats where complex, double-stranded target DNA is immobilized in a denatured, "hybridizable" form, the challenges of solution hybridization in a microtiter plate are significant. Complete denaturation of target, favorable thermal hybridization conditions to prevent rapid reannealing of target strands, and rapid hybridization of both detection and capture probes are equally important. These considerations led to the development of a robust, non-radioactive hybrid-capture method suitable for automated detection of herpes simplex virus (HSV) DNA in the 96-well format. This approach utilizes a pUC plasmid containing the UL-13 fragment of the HSV genome covalently attached to microtiter wells as a capture probe, and a digoxigenin-labeled PCR fragment containing 710 bp of the UL-15 fragment of the HSV genome as a detection probe. Direct thermal denaturation of target, capture and detection DNA in the capture plate was facilitated by the use of 3M guanidine isothiocyanate (GuSCN) in the hybridization buffer. Mineral oil overlays prevented evaporation and did not interfere with subsequent detection. Hybrids (captured target DNA annealed to digoxigenin-labeled probe) were detected by incubation with anti-digoxigenin HRP-conjugated antibody, followed by the addition of SuperSignal LBA chemiluminescent substrate. This allows for the identification of a potent anti-herpetic agent, which should manifest itself in this assay by a reduction in chemiluminescent signal corresponding to a decrease or inhibition of viral replication. Acyclovir, penciclovir, ganciclovir and HPMPC produced IC_{50} values that correlated well with those obtained by other methods. This HSV hybridization assay was designed to be a non-radioactive, microtiter-based system for the automated detection of HSV-1 or HSV-2 DNA in crude cell lysates or culture supernatants.

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Synthesis and Antiviral Activity of Carbocyclic Oxetanocin Analogues Bearing Fluorine

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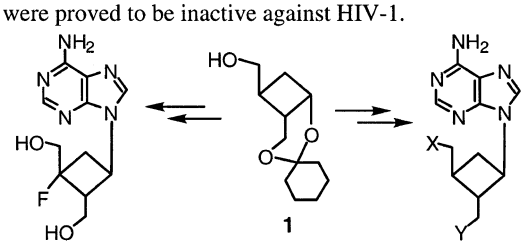
3'-Fluoro analogue of carbocyclic oxetanocin A (3'F-C.OXT-A, **2**) was prepared from *trans-cis*-2,3-bis(hydroxymethyl)-1-cyclobutanol analog (**1**) in 10 steps. To explore the role of hydroxy group the fluoromethyl congeners of carbocyclic oxetanocin A (**3**,**4**) were also prepared from **1**. 3'F-C.OXT-A showed some activity against HSV-l and HSV-2 with respective ED_{50} values of 2.72 and 1.33 μg/ml, which are seven times higher than those of acyclovir. It displayed potent activity against HCMV: at a concentration of 1.0 μg/ml, F-C.OXT-A completely inhibited HCMV replication, whereas ganciclovir showed only partial inhibition (58%). Ganciclovir was almost inactive at 0.1 μg/ml while F-C.OXT-A still showed 34% inhibition. Compounds **3**,**4** were proved to be inactive against HIV-1.



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Antiviral Activity of 2H-3-(4-Chlorophenyl)-3,4-Dihydro-1,4-Benzothiazine-2-Carbonitrile 1,1-Dioxide Against Human Betaherpesviruses

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The antiviral and antitumor activity of a new class of heteroaryl sulfones has been recently described [submitted, 2000]. We now report on the antiviral activity of 2H-3-(4-chlorophenyl)-3,4-dihydro-1,4-benzothiazine-2-carbonitrile 1,1-dioxide against human betaherpesviruses, i.e., cytomegalovirus (HCMV), human herpesvirus six (HHV-6) and human herpesvirus seven (HHV-7). The EC_{50} (50% antivirally effective concentration) based on microscopical evaluation of the virus-induced cytopathicity was 4.4 μg/ml in HCMV-infected human fibroblast cells, 0.91 μg/ml in HHV-6A-infected human T-lymphoblast HSB-2 cells and 1.6 μg/ml in HHV-6B-infected human T-lymphoblast MOLT-3 cells. At a concentration of 4 μg/ml, viral DNA synthesis was inhibited by ≥ 95% in HHV-6-infected cells, as estimated by Southern blot assay. In addition, the EC_{50} for inhibition of HHV-7-induced CD4 down-regulation in SUPT-1 cells was 5.7 μg/ml. These antiviral EC_{50} values were well below the cytotoxic concentration, the concentration producing microscopically visible alterations of normal cell morphology (MIC) being ≥ 20 μg/ml. Thus, this aryl sulfone derivative represents a novel non-nucleoside compound with strong and selective activity against all three human betaherpesviruses. The compound was also active against ganciclovir-resistant, foscarnet-resistant and ganciclovir/cidofovir-resistant mutants of HCMV, suggesting a different mode of action. Our ongoing experiments to measure transcription and/or translation of IE, E and L genes of HHV-6 or HCMV at different time points after infection, should reveal which step in the viral life cycle is inhibited by this compound.

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Activity of CADA Analogues Against Human Cytomegalovirus (HCMV) and Murine Cytomegalovirus (MCMV)

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We have evaluated the activity of several derivatives of CADA (cyclotriazadisulfonomide), a synthetic macrocycle, against HCMV and MCMV in two different fetal calf sera (FCS). CADA and some of its derivatives, i.e. MFS-025, MFS-067 and ARB-268 were active against HCMV (IC_{50} values ranging from 0.12 to 4 μg/ml) independent of the FCS used. However, the anti-HCMV activity of several derivatives was serum-dependent: IC_{50} values were in the range of 0.07-1.2 μg/ml when the assays were performed using the optimal FCS, and IC_{50} values were > 50 μg/ml in the presence of the other FCS. The activity of CADA analogues against MCMV was also dependent on the FCS used. The anti-HCMV activity of some of the CADA analogues was confirmed by different assays: virus yield, antigen expression as detected by flow cytometry and viral DNA synthesis as measured by hybridization. Time of addition experiments performed with several CADA analogues showed that the compounds affect an early stage of the HCMV replicative cycle. In addition, a mechanism of action different from that of GCV, CDV and PFA was also suggested by the fact that GCV^r, CDV^r/GCV^r and PFA^r strains of HCMV remained fully sensitive to different CADA analogues. Investigations are currently in progress to understand the dependence and mechanism of antiviral action of the CADA analogues, and to assess their efficacy against MCMV *in vivo*.

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The Antiviral Activity of Titanyl (TiO^{+2}) Sulfate Toward Human Cytomegalovirus

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Human cytomegalovirus (HCMV) is an opportunistic pathogen that is responsible for the life-threatening infections found in congenitally infected infants and immunocompromised patients. Retinitis, an infection caused by HCMV, is currently being treated by ganciclovir, foscarnet, or cidofovir. However, these antiviral drugs are not ideal due to their toxicity level and poor bioavailability. In addition, the long-term usage of these antivirals has lead to a positive selection of viral mutants that are resistant to these drugs. These various factors have initiated an active search for antiviral agents for HCMV that are both low in toxicity and high bioavailablity. Flow cytometry (FACS) drug susceptibility assay was used to measure the ability of the antiviral agent in blocking the spread of HCMV from one infected cell to the surrounding cells in a monolayer. This technique has permitted the testing of new antiviral agents with different modes of action seen in current drugs. The TiO^{+2} component of $TiOSO_4$ was found to have an IC_{50} of ~1 μM against the laboratory strain AD169 of HCMV. There was no toxicity associated with the use of $TiOSO_4$ towards the human foreskin fibroblast cells through microscope examination and cytotoxicity assays using Promega's CellTiter 96 aqueous non-radioactive cell proliferation assay.

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Sequencing of the Cytomegalovirus (CMV) UL97 Gene for Genotypic Antiviral Susceptibility Testing. N. S. Lurain[1] and S. Chou[2]. [1]Rush Medical College, Chicago, IL, and [2]Oregon Health Sciences University, VA Medical Center, Portland, OR.

The currently used phenotypic assays to detect CMV drug resistance are too time-consuming to provide therapeutically useful results. Recent advances in automated DNA sequencing techniques provide the rationale for developing this technology for rapid genotypic assays. Extensive sequence analysis of CMV clinical isolates has shown that the UL97 gene is the most frequent site of GCV drug resistance mutations, which occur as point mutations or short deletions at codons 460, 520 or 591-607. We have sequenced the complete UL97 coding region in a large number of phenotypically GCV-sensitive CMV clinical isolates to identify the baseline variability of this gene. No variability in these baseline sequences was found at codons 460, 520 or 591-607, except for codon 605. Changes in codon 605 have not been observed in GCV-resistant strains. Compared to the AD169 reference sequence, there are 89 variant nucleotide positions, 39 of which are present in more than one strain. Nucleotide changes are distributed across the entire coding region rather than occurring in clusters or patterns. Sequence identity among strains ranges from 98 to 100%. The corresponding amino acid sequences are even more highly conserved than the DNA sequences, indicating that the majority of nucleotide changes are synonymous. Compared to AD169 there are 16 amino acid residues that are substituted in at least one isolate, only 6 of which occur in more than one isolate. Using the sequence data from the drug-sensitive isolates combined with the known drug-resistance mutations, we have developed a genotypic assay based on direct DNA sequencing of PCR products amplified from viral genomic DNA extracts. Amplification of the C-terminal half of UL97 followed by two sequencing reactions allows detection of all presently known sites of GCV resistance attributed to this gene. Thus, we have identified primers and sequencing conditions for a rapid genotypic susceptibility assay. The assay can be extended to amplify the entire coding sequence of UL97 for identification of resistance mutations resulting from treatment with new antiviral agents that target this gene.

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Model for the Evaluation of Novel Antivirals to Prevent HCMV Dissemination

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During the active infection with human cytomegalovirus (HCMV), virus disseminate from the site of primary infection/reactivation via infected cells. In this process virus infected cells have to evade from the primary site, enter the blood system and after extravasation reach target organs where they infect susceptible cells. Extravasation as a crucial step in virus dissemination depends on interaction of cell adhesion molecules and/or specific viral proteins on infected cells with adhesion molecules of endothelium. We used persistently HCMV infected cells as well as cells transfected with human glycoproteins genes to study their importance for extravasation. Adhesion and penetration was analyzed by phase-contrast-reflection-interference-contrast-microscopy. Both persistently infected cells and cells transfected with gB and gH increased their adhesion and penetration to/through human umbilical vein cells (HUVEC). Monoclonal antibodies to HCMV gB and gH and hyperimmonoglubulin (Cytotect, Biotest, Germany) suppressed adhesion and penetration to HUVEC. These results show that virus dissemination via productively infected cells depends in part on HCMV gB and gH glycoproteins. This in vitro models provide opportunity for testing of novel antivirals develop to prevent HCMV dissemination.

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Positively charged proteins show anti-cytomegalovirus (CMV) activity dependent on the charge density.

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CMV infections are an important complicating factor in immunocompromised patients, such as transplant recipients and AIDS patients. Currently used anti-CMV drugs are nucleoside analogues (e.g. ganciclovir or cidofovir). Recently, we showed synergistic anti-CMV effects of the nucleoside analogues combined with lactoferrin (LF). LF, a cationic protein (pI = 8.5), is described to inhibit the cell entry of the virus. We now studied the anti-CMV characteristics of LF in more detail.

To study the influence of positive charge on the anti-CMV activity per se, we chemically cationized human serum albumin (cat-HSA) and betalactoglobulin (cat-bLG) by derivatization with ethylene diamine. The monomeric cat-HSA preparations had a pI ranging from 8.0 to 9.1. Using a recombinant human CMV labstrain (RC256), we showed that the *in vitro* anti-CMV activity of cat-HSA with pI 8.5 was comparable to that of lactoferrin. Furthermore, improved anti-CMV activity was observed when the pI of cat-HSA was increased showing a positive correlation of number of positive charges and anti-CMV activity (r^2 = 0.92). In addition, we assessed whether the size of the protein backbone affected the anti-CMV potency. Cationization of bLG (18 kD) was performed and resulted in a series of preparations with increasing substitution (all pI>9.3). In spite of the relative high pI, these compounds were much less potent as compared to cat-HSAs and LFs (±80 kD). The cationic proteins displayed no cytotoxicity *in vitro* (MTT assay) in concentrations up to 1 mg/ml.

Time of addition assays indicated that the cationic albumins interfere with the binding of CMV to the cell comparable to LF rather than having intracellular actions. The cationic proteins bound to the cells rather than to the virus. At molecular level, we were able to show binding of Cat-HSAs and LF to heparan sulphate proteoglycans.

In conclusion, the cell entry of CMV is inhibited by cationic proteins. The extent of inhibition is related to the degree of cationization and the size of the backbone protein. These compounds interfere with the docking of CMV on heparan sulphates at the cell membrane, which may have implications for therapeutic intervention with regard to CMV dissemination.

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Inhibition of p38 mitogen-activated protein kinase prevents human cytomegalovirus-induced upregulation of IL-8 and MGSA/Groα

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H.W. Doerr, J. Cinatl Jr.

Institute of Medical Virology Johann Wolfgang Goethe University Frankfurt/M, Germany;

Human cytomegalovirus (HCMV) infection is associated with excessive proinflammatory responses such as cytokine/chemokine production or upregulation of adhesion molecules on the host cells. These molecules may be regulated by mitogen activated protein kinase (MAPK) p38 which is induced during early and late times of HCMV infection. In the present study we observed effects of specific p38 MAPK inhibitor SB203580 on HCMV induced expression of intercellular adhesion molecule-1 (ICAM-1) and chemokines interleukin-8 (IL-8) and melanoma growth-stimulating activity (MGSA)/Groα. SB203580 treatment (20 μM) of human foreskin fibroblasts (HFF) infected with AD169 HCMV strain did not influence upregulation of the chemokines both at mRNA and protein levels 4h post infection as measured by RT PCR and ELISA, respectively. However, significant suppression of HCMV induced upregulation of both IL-8 and MGSA/Groα was observed in HCMV cultures treated with SB203580 for 24 and 72h post infection. Moreover, SB203580 suppressed induction of chemotactic activity in supernatants of infected cells as measured by transendothelial migration assay. SB203580 had no effects on HCMV induced ICAM-1. The results demonstrated that treatment of HCMV infected cells with p38 MAPK inhibitor significantly suppresses IL-8 and MGSA/Groα production as well as chemotactic activity for neutrophils in supernatants of infected fibroblasts. Thus, inhibition of p38 MAPK may be an important strategy for suppression of harmful inflammatory responses to HCMV infection.

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Carbocyclic 5'-Nor Guanosine.

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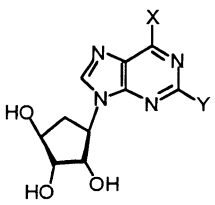
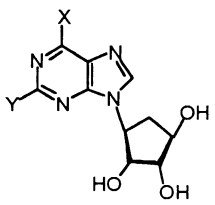
For the last decade, we have been investigating the antiviral properties of carbocyclic nucleosides lacking the 5'-methylene (5'-nor carbanucleosides). These efforts have proven fruitful with the two enantiomers of 5'-noraristeromycin (**1** and **2**) being the most promising. In that regard, **1** has shown activity towards, particularly, cytomegalovirus, measles, and the orthopoxviruses while **2** is effective towards hepatitis B virus. To determine if the antiviral characteristics of **1** and **2** extends to the guanine series (**3** and **4**), these analogs were prepared and evaluated. The only activity found was for **3** towards Epstein-Barr virus in both the VCA Elisa (EC_{50} 0.56 μg/mL; acyclovir EC_{50} 2.1 μg/mL) and DNA hybridization (EC_{50} 1.1 μg/mL; acyclovir EC_{50} 0.3 μg/mL) assays with no accompanying toxicity shown for the host Daudi cells. This research has been support by funds from the DHHS (AI 85347) and this assistance is appreciated. The assistance of Drs. Tseng, Kern, Korba and De Clercq in obtaining the antiviral data is also acknowledged.



1, X=NH_2; Y=H
3, X=OH; Y=NH_2



2, X=NH_2; Y=H
4, X=OH; Y=NH_2

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Highly Potent and Selective Inhibitors of Varicella Zoster Virus Based on Aryl Substituted Bicyclic Furo Pyrimidines.

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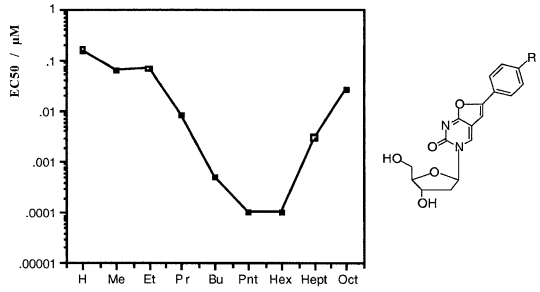
+ Rega Institute for Medical Research, Leuven, Belgium.

We have recently reported the discovery of an entirely new category of potent antiviral agents based on novel deoxynucleoside analogues with unusual bicyclic, fluorescent base moieties, bearing long (C8-C10) alkyl side-chains.
We now report that the introduction of a phenyl ring as part of the side-chain moiety leads to a >100-fold increase in antiviral activity, yielding compounds with EC_{50} values ≤0.1 nMol. A clear correlation exisits between alkyl(aryl) chain length [and thus lipophilicity] and anti-VZV activity:



The lead compounds are ca. 30,000 times more potent than acyclovir vs. VZV, and with no detectable in vitro cytotoxicity. In the presentation we will describe the discovery, synthesis, in vitro profile, SAR and mechanism of action of these novel agents.

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Strongly fluorescent tricyclic analogues of acyclovir.

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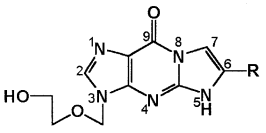
We have recently reported that transformation of acyclovir into its tricyclic derivative TACV {1,N^2-ethenoacyclovir, 3,9-dihydro-3-[(2-hydroxyethoxy)methyl]-9-oxo-5*H*--imidazo[1,2-*a*]purine, **1**} substituted in the 6 position with some aryl groups may lead to fluorescent analogues, e.g. 6-(4-methoxyphenyl)TACV (**2**), that showed similar antiherpetic potency as that of the parent acyclovir. In the search for analogues of **2** having more intense fluorescence and improved solubility and/or being prodrugs, we synthesized 6-(4-hydroxyphenyl)TACV (**3**), 6-(4-aminophenyl)TACV (**4**) and their derivatives **5-8**.



R	R
1 H	**5** 4-CH_3COOPh
2 4-CH_3OPh	**6** 4-PhOCOOPh
3 4-HOPh	**7** 4-PhOCONHPh
4 4-H_2NPh	**8** 4-$H_2NCONHPh$

The synthesis involved preparation of the appropriate α-bromoketones and their condensation-cyclization reactions with acyclovir. Quantum yield of fluorescence being below 10% in the earlier described analogues was in the range of 40-65% for the newly prepared compounds. Compounds **3**,**5**,**6**,**7** and **8** were evaluated for their antiviral activity and found active against herpes simplex virus (HSV-1 and HSV-2) at concentrations that were similar or higher than for acyclovir.

(Supported by KBN grant 4PO5F 00516).

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In vitro Selection of Drug-Resistant Varicella-Zoster Virus (VZV) Mutants: Differences between Acyclovir and Penciclovir ?

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VZV mutants were isolated under pressure of different classes of compounds, i.e. acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV), brivudin (BVDU), sorivudine (BVaraU), 2-phosphonylmethoxyethyl (PME) derivatives of adenine (PMEA) and 2,6-diaminopurine (PMEDAP), 3-hydroxy-2-phosphonyl-methoxypropyl derivatives of adenine (HPMPA) and cytosine (HPMPC) and foscarnet (PFA). Drug-resistant virus strains were obtained by serial passage of the OKA strain in human embryonic lung fibroblasts. The number of passages required to obtain the different drug-resistant mutants was significantly different: for the ACV^r, $BVDU^r$ and $BVaraU^r$ strains < 10 passages; for the PCV^r and PFA^r strains ~ 30 passages; for the $PMEA^r$ strain ~ 50 passages; the selection of GCV^r, $HPMPC^r$, $HPMPA^r$ and $PMEDAP^r$ strains has not been successful after about 75 passages over a period of 2 years. ACV^r, $BVDU^r$ and $BVaraU^r$ strains showed cross-resistance to all drugs that depend on phosphorylation by the viral TK (i.e. ACV, GCV, PCV, BVDU and BVaraU), suggesting an alteration at the TK gene level. These mutants remained sensitive to PMEA, PMEDAP, HPMPA, HPMPC and PFA. Interestingly, the PCV^r strain was resistant not only to PCV, but also to PMEA, PMEDAP, PFA and ACV, suggesting an alteration at the level of the viral DNA polymerase; the PCV^r strain was not resistant to BVDU, BVaraU, GCV, HPMPC and HPMPA. In contrast with the VZV mutants, HSV-1 mutants selected under the pressure of ACV or PCV showed a similar pattern of drug-susceptibility profile: resistance to TK-dependent drugs and sensitivity to PMEA, PMEDAP, HPMPA, HPMPC and PFA, suggesting mutations at the viral TK gene. Our results indicate that ACV and PCV may select different forms of VZV mutants *in vitro*; whether this is also the case *in vivo* should be further investigated.

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Determination of acyclovir in human plasma using solid phase extraction (SPE) and micellar electrokinetic chromatography (MEKC).

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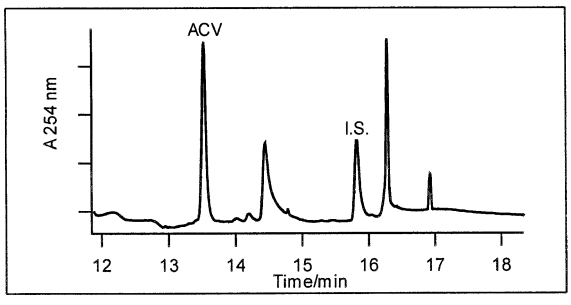
A rapid, simple, and sensitive assay for the determination of acyclovir has been developed using solid phase extraction (SPE) and micellar electrokinetic chromatography (MEKC) using 3-propylxanthine as an internal standard. The SPE was performed using a novel polymeric reversed-phase sorbent, Oasis HLB extraction cartridge and produced a three-fold concentration of acyclovir from 600 µl plasma samples. In MEKC, the effects of buffer ionic strength, pH, conductivity, additives, and organic solvents were studied in order to maximize separation as well as sensitivity via large volume sample introduction and sample stacking. Using this method, interfering plasma matrix peaks were completely separated from acyclovir (resolution > 2) and limit of quantitation (LOQ) of 20 ng/ml was obtained using UV detection at 254 nm. This method is a viable alternative to high-performance liquid chromatography because of its high separation efficiency and high sensitivity, ease of operation, rapid analysis, low consumable expenses, and ease of adaptability for other nucleoside analogues.



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Protection Against HSV-1 in Mice Following Immunization with Recombinant HSV-1 Glycoprotein D (gD) and a Semisynthetic Triterpenoid Saponin Adjuvant.

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We have previously demonstrated the superior ability of the aldehyde-containing semisynthetic triterpenoid saponin vaccine adjuvant GPI-0100 to be low in toxicity, maintain its stability, and promote a Th1-type immunity including IgG2a and IgG2b antibody and cell-mediated immunity (lymphoproliferation, IFN-γ production, and generation of CTL activity) in mice using ovalbumin as a noninfectious test antigen. In the current study, BALB/c mice were immunized with recombinant HSV-1 gD using the conventional adjuvant alum or GPI-0100 and then challenged with a lethal dose of HSV-1 to determine the ability of these regimens to confer protection. Survival results are shown below:

Treatment	% Survival
PBS	12.5
gD (5 μg) only	0
gD (5 μg) + alum (200 μg)	30
gD (5 μg) + GPI-0100 (100 μg)	100

GPI-0100 was superior in eliciting a Th1-type response (IgG2a antibody and IFN-γ production). Additional studies are underway to further characterize this system for the clearance or persistence of virus and the ability of treatment with vaccines using GPI-0100 as immunostimulatory adjuvant to affect HSV infection.

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Preclinical evaluation of docusate as protective agent from herpes simplex viruses (HSV)

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Docusate is a sulfated surfactant and, as such, may inactivate viral pathogens by disrupting viral envelopes and/or denaturing proteins. Accordingly, the *in vitro* efficacy and toxicity of docusate (Zorex™; Meditech Pharmaceuticals, Inc.) against HSV were evaluated. Docusate was effective *in vitro* against wild type and drug-resistant strains of HSV-1/-2 with $EC_{90\text{-}100}$ of approximately 0.005% (w/v), and was slightly less toxic to uninfected cells compared with both SDS and sarcosine (docusate CC_{50} ~0.01% vs. SDS/sarcosine ~0.005% after 2 days incubation). The cytotoxicity profiles of docusate were time- and dose-dependent and thus associated with the frequency of use. Kinetics of inactivation examined by pre-mixing virus and drug in a time-course experiment demonstrated that docusate could reach its $EC_{90\text{-}100}$ within 30 min. Docusate pretreatment of cells was associated with a 45% reduction in infectivity of those cells, despite a triple washing procedure. Once infected, an approximate 30% plaque reduction was observed with treatment. Our studies demonstrate that docusate has favorable *in vitro* characteristics warranting consideration for use as a topical microbicide in the prevention of sexually transmitted infections.

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Second-Generation Dendrimers as Topical Microbicides Against Genital HSV-2 Infection: Studies *in vitro* and in a Mouse Model

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[1]Children's Hosp. Med. Center, Cincinnati, Ohio; [2]Viridae(USA), Cincinnati, Ohio, [3]Starpharma Ltd, Parkville, Australia.

Dendrimers are highly branched macromolecules synthesized by the addition of repeat units to a central core molecule. Selected dendrimers have shown *in vitro* activities against a broad spectrum of sexually transmitted viruses including HBV, HIV and HSV. Because of their broad spectrum, high therapeutic indices and mechanisms of action, they are candidates for use as topical microbicides in preventing sexually transmitted viral infections. We have previously shown that a number of dendrimers could prevent genital HSV-2 infection in mice when applied intravaginally before virus challenge. Here, we have assessed the *in vitro* and *in vivo* anti-HSV-2 activity of four second-generation dendrimers with improved synthesis and stability characteristics. All four dendrimers were active *in vitro* when added to cells one hour before the virus (IC_{50}, values of 0.4-0.7 μg/ml). Efficacy was reduced when the dendrimers were added to already infected cells (IC_{50} values 2.7-26.2 μg/ml). None of the dendrimers showed toxicity (CC_{50} values >10004μg/ml). For *in vivo* studies, mice were administered 15μl of a 100mg/ml dendrimer solution intravaginally 20 seconds before HSV-2 challenge. Three of the compounds provided significant protection against infection. BRI-7013 (23/23 vs 0/32 PBS; $p<0.001$), BRI-7015 (23/28;$p<0.001$) and BRI-7032 (26/28; $p<0.001$). Further when the concentration of BRI-7013 was reduced to 10mg/ml 83% of animals were still protected against HSV-2 infection (10/12 vs 0/12 PBS; $p<0.001$) and even at 1 mg/ml significant protection was seen (6/12 vs 0/12 PBS; $p<0.05$). Formulation of second-generation dendrimers are in progress. We believe that this will improve efficacy and that further studies with these molecules are warranted.

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Virus replication in various tissues of mice infected with HSV-1 cutanoeusly for mouse zosteri model.

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Mouse zosteri model is clinically relevant in that there is a primary infection in the skin, movement of virus in the peripheral nervous system and spread back to the skin to infect the whole dermatome. The secondary infection has many similarities with recurrent infection in the natural host. To understand the viral pathogenesis and antiviral efficacy of acyclovir, various tissues were isolated from the virus-infected mice and the virus titer of each tissue was measured and compared to clinical aspects. Five week-old BALB/c mice were cutaneously infected in the lumber region with HSV-1. Acyclovir-treatment by intraperioneal injection twice daily for 7 days commencing 1 h after infection. Lesions developed at the infection site and then spread laterally to involve the hindlimb paralysis in the untreated F-infected mice. Skins at the primary and the secondary infection sites, dorsal root gaglia, spinal cords, brain stemps, cerebra, cerebella, trigeminal ganglia were isolated from 2 mice per group every day up to 2 weeks p.i. and the virus titers were measured by plaque assay in Vero cell culture system. There were strong correlation with virus titers and clinical syndrome, such as lesion socres at secondary infection site of skins, hindlimb paralysis and virus appearance in brain tissues, etc.

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Successful antiviral and immune therapy of severe Epstein-Barr virus (EBV)-induced lymphoproliferative syndrome after bone marrow transplantation (BMT). Report of two cases.

A. Meerbach[1], B. Gruhn[2], F. Zintl[2], and P. Wutzler[1]
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EBV-associated lymphoproliferative disease is a serious complication especially in patients receiving BMT. Here, we report two patients who developed EBV-induced lymphoproliferative disorder 60 days following unrelated BMT. To detect the active EBV infection as well as to follow up the effect of therapy a semiquantitative PCR assay for EBV DNA in plasma and peripheral blood mononuclear cells was used. The method bases on a nested PCR assay using primers of the structural protein region p23 and an end-point dilution.

Patient 1 was diseased with fever and cervical lymph node swelling. The high EBV load in plasma and peripheral blood mononuclear cells could be reduced dramatically after antiviral treatment with cidofovir and aciclovir in combination with hyperimmune globulin and stop of immunosuppression. He gradually improved and recovered completely within 4 weeks.

Patient 2 became ill with fever, inguinal lymph node swelling and tonsillitis. The PCR assay revealed extremely high amounts of EBV DNA in plasma and peripheral blood mononuclear cells. After therapy with cidofovir, ganciclovir, anti-CD20 monoclonal antibodies, donor lymphocytes as well as discontinuation of immunosuppressive drugs the viral burden decreased remarkably and reached normal data accompanied with complete recovery of clinical signs.

In conclusion, two cases of severe lymphoproliferative disorder with EBV infection following BMT were successfully treated with combined antiviral and immune therapy. The semiquantitative PCR assay for evaluation EBV genome copy numbers in plasma and peripheral blood mononuclear cells is the method of choice for laboratory diagnosis of EBV infection as well as monitoring the response to therapy in immunosuppressed patients.

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Activity of adenosine-N[1]-oxide derivatives against variola and other orthopoxviruses. R. O. Baker[1]*, C. D. Kwong[2], J. A. Secrist III[2], and J. W. Huggins[1]. [1]US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD, and [2]Southern Research Institute, Birmingham, AL.

Orthopoxvirus infections, including that of variola (VAR), the causative agent of smallpox, are of significant concern to both health and defense officials. In order to evaluate potential drugs useful in combating orthopoxvirus infections, we have examined the sensitivity of four isolates of VAR, as well as monkeypox (MPX), cowpox (CPX) and vaccinia (VAC) viruses to a series of 11 drugs. These drugs are chemically-modified derivatives of adenosine-N[1]-oxide (ANO), a drug known to have activity against VAC, and are believed to be inhibitors of viral protein synthesis. We have employed *in vitro* assays using live virus handled under biosafety level 4 containment in a 96-well plate format to determine drug efficacy and toxicity in several monkey and human cell lines. These assays use neutral red uptake to determine cell viability after infection and/or drug treatment. All drugs tested showed low cytotoxicity (TC_{50} = 114 - ≥370 μM). Derivative compounds showed varying levels of activity that fell into three main groups. Six drug derivatives were highly active, with IC_{50} values for VAR between 0.07 and 1.0 μM, and between 0.37 and 6.7 μM for the other viral species. These six drugs had therapeutic indices from 28 to more than 3300. One drug showed intermediate activity, with an IC_{50} ranging from 1.1 to 5.6 μM for isolates of VAR, and 21.8 to 38.9 μM for the other viral species. The remaining four derivatives showed the least activity, with IC_{50} values for VAR from 2.8 to 101 μM, and 94 to ≥270 μM for other viruses. There exist significant differences in sensitivity to some of the drugs tested; ≥100-fold among VAR, MPX, CPX and VAC viruses, and up to 20-fold among the four VAR isolates. These drugs also showed cell line-dependent variations in IC_{50} values. We conclude from these data that at least several derivatives of ANO are excellent candidates for further development as anti-pox agents.

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Efficacy and Safety of Brivudin in the Treatment of Herpes Zoster

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Two large randomized, double-blind multicenter trials were conducted to evaluate the efficacy and safety of oral brivudin 125 mg once daily for 7 days in the treatment of herpes zoster. Comparison to acyclovir: the first study compared the efficacy of brivudin with acyclovir 800 mg five times daily in 1227 immunocompetent herpes zoster patients, aged 18 years or older. Treatment started within 48 hours after the first vesicular eruption. In the ITT population (N=1224), brivudin proved to be superior to acyclovir in reducing the time to the last eruption of new vesicles (RR: 1.13, *P*=0.01). For this primary endpoint, superiority of brivudin over acyclovir could also be shown in the subpopulation of patients aged 50 years or older (RR: 1.16, *P*=0.02). In patients with new vesicle formation after start of treatment, the time to stop of new vesicle formation was 27.9 hours with brivudin and 32.3 hours with acyclovir (*P*=0.03). The time to resolution of acute pain was the same for both drugs. Similar results were obtained in the PP population. Adverse events with a possible or probable relationship to study medication occurred in 8% of the brivudin patients and 10% of the acyclovir patients. Comparison to famciclovir: the second study compared brivudin with famciclovir 250 mg t.i.d. in 2027 immunocompetent patients aged 50 years or older. Treatment started within 72 hours after rash onset. In the ITT population of patients with new vesicle formation after start of treatment, the mean time to the last new vesicular eruption was the same with both study drugs (brivudin: 40.0 hours, famciclovir: 40.4 hours; RR: 1.01, CI: 0.90-1.12). No difference between the two study drugs could be detected for the time to full crusting and complete loss of crusts. Adverse events with a possible, probable, or not assessable relationship to study medication occurred in 11.8% of the brivudin patients and in 10.1% of the famciclovir patients. Conclusions: Brivudin is significantly more effective than standard acyclovir and as effective as famciclovir in alleviating acute signs and symptoms of Herpes zoster. The safety profile of brivudin is good and well comparable to that of acyclovir and famciclovir. Thus, brivudin is a valuable new option in Herpes zoster therapy, with the additional advantage of a once daily treatment schedule.

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Vaccinia Virus Respiratory Infection Model in Mice for Antiviral Studies. Donald F. Smee, Kevin W. Bailey, and Robert W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, Utah, USA.

Intranasal infection of BALB/c mice with the WR strain of vaccinia virus leads to profound weight loss, pneumonia, and death. This model was used to study the efficacy of cidofovir in treating the infection. Subcutaneous injections of 100 and 30 mg/kg/day given on days 1 and 4 after virus challenge reduced mortality by 60-100%. Lung consolidation scores and lung weights were also reduced in treated groups. Lung virus titers on days 2-5 were reduced no more than 10-fold by these treatments, however. A moderate improvement in drug efficacy occurred following daily treatments for 5 days with 30 mg/kg/day. The efficacy of cidofovir also increased as the virus challenge dose decreased. Comparisons were made between the vaccinia infection model and the similar cowpox virus respiratory infection model. Both infections led to high virus titers in lungs and nose/sinus tissues on day 5. However, vaccinia virus in blood was 300,000-fold higher than that found in cowpox virus infected mice. Significantly higher vaccinia virus titers (25- to 630-fold) were also present in nine other organs and tissues. Arterial oxygen saturation declined as death approached and in relation to the severity of the infection. Intraperitoneal cidofovir treatments of 30 and 10 mg/kg given on days 1 and 4 of the infection in mice given varying cowpox or vaccinia virus challenge doses showed that the drug was similarly effective against both infections, taking into account the increased virulence of vaccinia virus and adjusting for the virus challenge dose. Although it has been known for many years that this strain of vaccinia virus can cause a lethal infection by intranasal route, its application to antiviral therapy represents a new model for studying anti-orthopoxvirus agents. Supported by Contract NO1-AI-85348 from the Virology Branch, NIAID, NIH.

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Systemic and Cutaneous Infections of Mice with Vaccinia and Cowpox Viruses and Efficacy of Cidofovir. D.J. Collins, D.C. Quenelle and E.R. Kern. The University of Alabama Sch. of Med., Birmingham, AL, USA.

Orthopoxviruses, including variola and monkeypox, pose risks to human health through natural transmission and potential bioterrorist activities. Since vaccination is currently not feasible for control of these infections, there is a renewed effort in the development of antiviral agents. An important part of this development is the need for small animal models for the evaluation of new therapies directed against orthopoxvirus infections. We have utilized BALB/c or SCID mice inoculated i.p. with Vaccinia virus (VV) or Cowpox virus (CV) as models for systemic poxvirus infections. In addition, we have inoculated SKH-1 mice by the orofacial route as a model for cutaneous disease. Since both VV and CV are sensitive to cidofovir (CDV) in vitro, we have determined its efficacy in each of the model infections. BALB/c mice were inoculated i.n. with VV or CV and treated with 6.7, 2.2 or 0.7 mg/kg of CDV beginning 48, 72 or 96 h post-inoculation. In VV infections, the 6.7 mg/kg dose at +48 and +72 h had 0% mortality and at +96 h had 40% ($p<0.001$). In CV infections, the 6.7 mg/kg dose at +48 and +72 had only 40% mortality ($p<0.01$) and 53% mortality ($p<0.05$), respectively. SCID mice were inoculated i.p. with VV or CV and treated for 7-30 days with 20, 6.7 or 2.2 mg/kg of CDV beginning 48, 72 or 96 h post-inoculation. All mice eventually died after cessation of treatment, but significant delays in time to death occurred in most treated groups. For the cutaneous infection, SKH-1 mice were inoculated with VV or CV onto lightly abraded epidermal areas on the snout. Orofacial and body lesions were scored daily and orofacial lesion swabs were obtained on 1,3,5,7,9,11,13 and 15 days post infection for viral titration. Rash developed on the orofacial region in 100% of mice beginning on days 4-5 and focal skin lesions developed on the body by day 9 in ~40% of mice. Peak orofacial and body lesion scores occurred on days 9-11. Depending on inoculum size, virus titer day area under the curve values ranged from 14.9 to 35.7 for VV and 8.4 to 46.1 for CV. No mortality was observed. Animals were next inoculated on the snout with CV and treated i.p. with 20, 6.7 or 2.2 mg/kg of CDV beginning 24 h post-inoculation and results are pending. These studies confirm those of others that VV and CV can be utilized for infection of mice and are susceptible to treatment with CDV. We have also demonstrated that these viruses can cause cutaneous infection in SKH-1 mice. Our results suggest that these models should be very useful in evaluating new therapies for orthopoxvirus infections.

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Protective Effect of 5-Iodo-2'-deoxyuridine (IDU) against Vaccinia Virus Infections in SCID Mice

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There is a concern that there may be unregistered stocks of smallpox that can be used for bioterrorism or biological warfare. A smallpox outbreak occurring today in a highly mobile and susceptible population (all children and many adults have not been vaccinated) would likely spread widely before effective measures could be taken. In December 1999, the "WHO advisory committee on variola research" urged to develop strategies to treat smallpox infections should they reappear. Also for the monkeypox disease it would be important to have an effective treatment at hand. We previously reported the protective effect of cidofovir (CDV) and of the N-7 substituted acyclic nucleoside analog 2-amino-7-[1,3-dihydroxy-2-propoxy)methyl]purine (compound S2242) against vaccinia virus (VV) infections in a tail lesion model in immunocompetent mice and in a lethal systemic infection model in SCID mice. Almost 25 years ago, IDU was shown to be active in the VV tail lesion model. Protective activity of IDU in a model for lethal orthopoxvirus infections has never been demonstrated. We have now studied the effect of 5-iodo-2'-deoxyuridine (IDU) on a lethal VV infection in SCID mice that had been infected intranasally. Untreated mice became sick and died 20.8 ± 3.1 days after infection. Histological examination of the lungs of infected animals showed evidence of viral pneumonitis and pulmonary hemorrhage. Treatment with IDU (5 consecutive days at 150mg/kg/day and, following a 2 day interval, 5 consecutive days at 75 mg/kg/day) delayed-virus induced mortality by 15 days (mean day of death 35.8 ± 6.7, $p< 0.0001$) with a reduction of the severity of the pneumonitis and pulmonary hemorrhage. Investigations are in progress to define the activity of other treatment schedules. We also demonstrated that IDU, like CDV and compound S2242, inhibits *in vitro*, in a dose-dependent way VV DNA synthesis (EC_{50}: 0.3 µg/ml, 0.2 µg/ml and 0.2 µg/ml for IDU, CDV and S2242, respectively). Our data indicate that IDU may be a candidate drug for the treatment of (ortho)poxvirus infections.

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In Vitro Evaluation of Compounds Against West Nile Virus. J. D. Morrey, D. F. Smee, and R. W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, Utah, USA.

The recent West Nile virus (WNV) outbreak in the United States has necessitated a search for potentially effective therapeutic agents. Chemotherapeutic approach to treatment may be a good strategy because the virus infection is typically not a long duration and antiviral drugs have been identified to be effective against other Flaviviruses. A total of 22 test compounds was evaluated against an infection induced by a New York isolate of WNV in Vero cells. Five active compounds have been identified with 50% effective concentrations (EC_{50}) less than 10 µg/mL and with a selective index (SI) of greater than 10. Known inhibitors of OMP decarboxylase and IMP dehydrogenase involved in the synthesis of GTP, UTP and TTP were most effective as compared with adenosine analogues. The most effective compounds were 6-azauridine (also 6-azauridine triacetate), selenazofurin, pyrazofurin, 2-thio-6-azauridine and cyclopentenylcytosine (CPE-C). These antiviral responses of the recent New York isolate were compared with the responses of a 1937 Uganda isolate from the American Type Culture Collection (strain B956).
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SULFATED SIALYL LIPID (NMSO3) IS A POTENT ANTI-HUMAN ROTAVIRUS INHIBITOR IN VITRO AND IN VIVO

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Anti-human rotavirus (HRV) activity of sulfated sialyl lipid (NMSO3) was examined by MTT assay. NMSO3 inhibited the replication of Wa strain (G1) of RV in MA104 cells at EC_{50} of 0.82µg/ml with CC_{50} of 153 (SI=186). Exposure of NMSO3 to HCl pH2.0 for 30 min exhibited no loss of anti-RV activity. Time of addition experiments revealed that NMSO3 inhibited the adsorption of four serotypes of RV to MA104 cells. Furthermore, virus binding assay with radiolabeled rotaviruses revealed that NMSO3 inhibited the virus binding to MA104 cells. Prophylactic oral administration of NMSO3 (10µg x3/day, 4 days) to suckling mice starting 30min before inoculation of MO strain ($3x10^6$pfu/mouse) almost prevented the development of diarrhea. Administration of less amount of NMSO3 (2 and 0.4µg x3/day) had little effect for preventing diarrhea. Administration of NMSO3 (10µg x3/day) starting 6 h after virus inoculation prevented diarrhea, but that starting 12 h after inoculation did not. The mean antibody titer of mice which received NMSO3 at 10µg x3/day for 4 days was significantly lower than that of non-treated, infected mice, suggesting that NMSO3 inhibited the viral replication in the intestines of the treated mice followed by low antibody responses. Since intestinal uptake of NMSO3 is very low (0.5%) and there is no acute toxicity of NMSO3 in mice (1500mg/kg, single intraperitoneal injection), NMSO3 is a promising candidate for the prophylactic and therapeutic treatment of HRV infection in humans.

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Lactobacillus rhamnosus HN001 Enhances Resistance to Rotavirus Infection in Mouse and Piglet Models

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Millions of people and animals suffer from infection such as diarrhea associated with rotavirus. Currently, there is no satisfactory strategy to control rotavirus infection. It has been reported that oral probiotics (lactic acid bacteria) can enhance immunity and resistance to intestinal pathogens. Recently, our laboratory has identified a new probiotic strain, *L. rhamnosus* HN001, and has further examined its protective effect against rotavirus in piglet and mouse models. *Piglet study*: Eighteen piglets were allocated into 2 groups. The test group was orally administered with HN001 (10^9 cfu/piglet/day); the control group did not receive probiotics. After one week of HN001 feeding, the animals were penned individually and weaned onto a weaner diet for two weeks; during that time they were susceptible to diarrhea. Compared to the controls, HN001-fed piglets had less severe diarrhea, associated with a lower level of rotavirus shedding, and maintained greater feed intake and liveweight gain ($P<0.05$). The blood cell phagocytic activity and lymphocyte mitogenic responses in the HN001-fed group were significantly higher than the control group. Enhanced resistance to weaning diarrhea was also accompanied by higher levels of mucosal antibody responses. *Mouse study:* After one-week acclimatization on a skim milk-based diet, 60 BALB/c mice were randomly allocated to two groups. The test group was orally administered 10^9 cfu/mouse/day of HN001, while the control group did not receive probiotics. After one week, animals were orally challenged with rotavirus (10^5 FFU/mouse). Protection was demonstrated by a lower level of fecal rotavirus shedding in the HN001 group, which was associated with higher specific and non-specific immune responses, in comparison with the controls. These results suggest that HN001 may have a protective effect against rotavirus and that immunostimulation induced by HN001 may play an important role in the protection.

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Recombinant Bispecific Single Chain Antibodies Specific Against Alphaviruses: Development and Characterization

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Bispecific antibody (BsAb) has been shown to be one of the most promising immunotherapeutics for the treatment of cancer as well as infectious diseases. BsAb that has dual specificity for both T lymphocytes and target antigen redirect cytotoxic T cells to mediate target cell lysis regardless of their initial T cell antigen specificity or major histocompatibility complex antigen. We have currently produced three different BsAb specific against murine T lymphocytes and Western Equine encephalitis virus (WEE) or Venezuelan Equine encephalitis virus (VEE) using genetic engineering. The construct is composed of single chain Fv (scFv) specific against murine CD3 (clone 2C11) joined together with WEE scFv (clone 11D2) or VEE scFv (clones 5B4D6 and A4A1) by a 23 amino acid residues. The bispecific antibodies could be successfully expressed as a 65 kDa protein in both mammalian and yeast expression systems with an estimated production yield of 1mg/L. Western blot analysis of the three different bispecific scFvs, 2C11x11D2, 2C11x5B4D6 and 2C11xA4A1, suggested a proteolytic sensitive site in the inter-scFv linker region. As a result, bispecific scFv produced in *P.pastoris* yielded a mixed products of 65 kDa and 32kDa. Expression in mammalian cells, on the other hand, produced bispecific scFvs with variable yield and stability. Comparative analysis of transiently transfected COS cells showed that 2C11x11D2 was produced as a stable 65 kDa protein while 2C11x5B4D6 and 2C11xA4A1 were produced at lower level with the evidence of the breakage of protein into 32 kDa subfragments. Thus, the studies suggested that each bispecific scFvs is unique in terms of its production yield and protein stability and will require its own optimization in order to obtain the useful amount of antibodies. These three bispecific antibodies are currently produced in large quantities for the evaluation of their biological properties in the treatment of alphavirus infection.

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Development and Characterization of a single chain Antibody -Biotin Tag against Western Equine Encephalitis Virus

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A recombinant single chain variable fragment (ScFv) antibody specific against Western Equine Encephalitis (WEE) Virus was developed and characterized. The ScFv was generated from 11D2 hybridoma producing anti-WEE antibody reactive to the E1 component of the viral envelope glycoprotein. VL and VH gene segment of 11D2 ScFv were generated and joined together with a (gly4ser)3 linker by PCR. The resulting ScFv with either the c-myc tag or the c-terminal peptidomimetic biotin tag was successfully expressed in a *P.pastoris* system. Individual plasmids containing the c-myc or biotin tag were tested and several of them were shown to drive ScFv expression. Western blot analysis of the ScFv using the anti c-myc antibody or streptavidin peroxides showed the presence of the bifunctional antibody. Purification of the ScFv with the two tags were successfully accomplished by affinity and T-gel purification protocols. The ScFv was demonstrated by ELISA to bind the WEE antigen with similar affinity when compared with the parental antibody. Thus, the ScFv with the biotin tag should be a useful immunodiagnostic tool for sensitive detection of the WEE virus.

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Effect of *Porphyridium* sp. Polysaccharide on Malignant Cell Transformation by Moloney Murine Sarcoma Virus

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The polysaccharide produced by the red microalga *Porphyridium* sp. was highly inhibitory for cell transformation of mouse fibroblast cells by an MSV-124 virus stock. This inhibition was most effective if the polysaccharide was added 2 h before or at the time of infection. The finding that the inhibition of cell transformation by MuSV-124 was reversible after removal of the polysaccharide suggested that *Porphyridium* sp. polysaccharide inhibited a late step after provirus integration into the host genome. Addition of the polysaccharide post-infection significantly reduced the number of transformed cells, but its effect was less marked than that obtained when the polysaccharide was added before or at the time of infection. These findings support the possibility that the polysaccharide may affect early steps in the virus replication cycle, such as virus absorption into the host cells, in addition to its effect on a late step after provirus integration.

Oral Session V: Retrovirus Infections II

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Dynamic of HIV Inhibition by Antiviral Drugs in Long-Term Cultures of Human Primary Macrophages.

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Objectives: Assessment of the long-term dynamics of HIV replication in cultures of primary macrophages (M/M), and evaluation of cumulative HIV production and inhibition under treatment with RT- or PR-inhibitors. Methods: In vitro HIV-infected M/M were long-term cultured and the cumulative antiviral activity of AZT or ritonavir (RTV) was evaluated. The cumulative HIV production (RNA, gag protein, and virus infectivity) was calculated by estimating the area under the curve of the virus production /10^6 cells (AUC), using the trapezoidal method. The Student's t test was used for statistical analysis. Results: Unspliced (US) and multiply-spliced (MS) RNA linearly increased starting from day 3 after infection, reaching at day 10 levels of 1.48×10^8 and 2.77×10^5 copies/10^5cells respectively. HIV-RNA production at day 50 was still 1.72×10^8 and 3.85×10^5 copies/10^5cells for US and MS respectively (MS/US ratio: 0.002). Genomic RNA and p24 Ag production in supernatants paralleled the production of US and MS, with levels at day 10 of 4.86×10^8 copies/10^5cells and of 2.41×10^4 pg/10^5 cells respectively. The AUC of p24 and genomic RNA at day 50 was 1.06×10^7 pg /10^6cells and 1.02×10^{10} copies/10^6cells respectively. Production on a per-cell basis averages 10^2 virus particles per day for each infected M/M. AZT (0.04 μM) inhibited HIV replication in M/M without detectable virus rebound up to day 50 of culture (79.6% inhibition of HIV US-RNA production compared to controls). The AUC of genomic RNA and p24 Ag at day 50 in AZT-treated M/M were 2.5×10^9 copies/10^6cells (73.8% inhibition; $p<0.01$), and 1.1×10^6 pg/10^6cells (88.9% inhibition; $p<0.001$) respectively. RTV (4 μM) was totally inactive upon HIV-RNA (either US or MS), but was somewhat effective upon virus infectivity (inhibition of 75%) and production of HIV protein (AUC of p24 Ag during RTV treatment: 6.9×10^6 pg/10^6cells, corresponding to an inhibition of 40.1%, $p=0.04$). Conclusions: Long-term and high-level HIV replication was demonstrated in M/M up to 50-days culture, thus supporting their role as HIV reservoir in the body. AZT effect upon virus production is substained and prolonged, while RTV is effective only at very high concentrations, thus suggesting an incomplete antiviral effect at physiological tissue concentration.

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Synthesis and Anti-HIV Activities of Neomycin B-Arginine Conjugate; A novel HIV-1 Inhibitor and Tat Antagonist

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Novel approaches for anti-HIV chemotherapy are much in demand and Tat targeting may comprise an important strategy. Since Tat is involved in multiple steps of HIV progression, it functions not only as an HIV gene regulator, but while secreted extracellularly, also as an immunosuppressor, activator of quiescent T cells for productive HIV-1 infection and a chemokine analog. Tat up-regulates the expression of chemokine receptors CXCR4 and CCR5, which serve as HIV-1 entry co-receptors. We have recently designed and performed a set of peptidomimetic TAR RNA binders, conjugates of arginine with aminoglycoside antibiotics (1-3). These relatively low molecular weight compounds are almost as efficient as the Tat peptide in their binding to TAR RNA. Aminoglycoside-arginine conjugates (AAC) inhibit HIV proliferation in infected cells, display low cytotoxicity and accumulate in mammalian cell nuclei (2) and also function as CXCR4 antagonists (4,5). We have also found that AAC inhibit HIV infection at different stages and have shown, for the first time, the ability of AAC to compete with several HIV Tat intracellular (transactivation) and extracellular (i.e. immunosuppression) functions (5). To the best of our knowledge, AAC are the only compounds found so far that inhibit extracellular activities of Tat. To this end, our data point to NeOR (hexa-arginine neomycin), as an attractive lead compound, capable of interefering with different stages of T- and M-tropic HIV infection and AIDS pathogenesis.

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Mechanisms of Anti-HIV Action of SJ-3366: Effects on Reverse Transcriptase and Cell Surface Events

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SJ-3366 is a highly potent inhibitor of both HIV-1 and HIV-2. Against HIV-1, SJ-3366 inhibits both laboratory and clinical strains of virus with IC95 values in the low nanomolar range. HIV-2 strains are inhibited at concentrations approximately 100-fold higher. We have performed a variety of mechanistic assays to address the means by which SJ-3366 inhibits the replication of these diverse viruses and have demonstrated that the compound acts through at least two distinct antiviral mechanisms of action. Against both HIV-1 and HIV-2, SJ-3366 inhibits the initial events of infection by interfering with a post-attachment, self surface event involved in virus entry and subsequent infection. SJ-3366 does not interfere with the interaction of CD4 and gp120 or virus attachment to the surface of the target cell, but does interfere with the ability of the virus to penetrate the cell membrane. In cell based assays SJ-3366 interferes with the fusion of infected and uninfected cells. We have shown in virus/cell complex assays that SJ-3366 may act similarly to the peptide T20 by binding to a tertiary complex formed upon interaction of the virus and the cell. This self surface mechanism is the primary inhibitory activity of SJ-3366 against HIV-2 and the secondary (in terms of potency) mechanism of action against HIV-1. SJ-3366 acts as a typical nonnucleoside RT inhibitor against HIV-1. The compound acts at low nanomolar concentrations to inhibit HIV-1 RT in biochemical assays, exhibiting Ki values of 1-3 nM, but is totally inactive against HIV-2 RT in the same assays. Evaluation of the activity of SJ-3366 against a variety of NNRTI-resistant strains has demonstrated the specificity of the interaction of SJ-3366 in the hydrophobic NNRTI binding pocket. Resistant strains of both HIV-1 and HIV-2 have been selected in cell culture which provide further evidence of the two mechanisms of antiviral action, with amino acid changes occurring in both env and RT with HIV-1 and only in env with HIV-2. Structure activity assays have been performed to attempt to assign these two antiviral activities to distinct features of the molecule and these data suggest a complex interaction of molecular features occurs and may hint at the possibility of an additional mechanism of action for SJ-3366 or synergistic interactions between the RT and surface active antiviral activities.

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Removal of Nucleoside and Nucleotide RT Inhibitors by the ATP-Dependent Chain-Terminator Removal Mechanism

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We have examined whether HIV RT-mediated removal of nucleoside chain-terminators by ATP-dependent chain-terminator removal (a mechanism related to pyrophosphorolysis using ATP as the acceptor molecule) contributes to NRTI resistance. Recombinant HIV-1 wild-type RT and mutant RT enzymes containing the zidovudine/thymidine analog resistance mutations were analyzed for activity in removing the chain-terminating nucleosides, zidovudine and stavudine, and the nucleotide analog, tenofovir, in the presence of physiological concentrations of ATP. The wild-type RT enzyme demonstrated efficient removal of zidovudine and stavudine, and increased removal of these chain-terminators was observed with the mutant RT enzymes compared to wild-type RT. However, wild-type and mutant RT enzymes showed minimal removal of tenofovir. In a further analysis, the addition of ATP and the next complementary dNTP resulted in a significant reduction in the specific removal of stavudine, consistent with the more moderate changes in susceptibility associated with stavudine as compared to zidovudine. Our results confirm that ATP-dependent chain-terminator removal is a potential mechanism of NRTI resistance and that this effect for stavudine can be moderated by the presence of dNTPs. Tenofovir is only minimally removed under these conditions, consistent with the minor changes in susceptibility to tenofovir of the zidovudine/thymidine analog-resistant HIV RT mutants. The inability to remove tenofovir efficiently by this mechanism of resistance may contribute to the durable anti-HIV activity observed in nucleoside-experienced patients treated with its oral prodrug, tenofovir disoproxil fumarate.

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Structure and activity relationship for tenofovir amidates, novel intracellular prodrugs of tenofovir

GX He, EJ Eisenberg, T Cihlar, H Chapman, WA Lee
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Background. Tenofovir is an acyclic nucleotide analog, which has potent activity against HIV. An oral phosphodiester prodrug of tenofovir, tenofovir disoproxil fumarate (TDF), is currently in Phase III clinical trials for the treatment of HIV. In plasma, tenofovir is the only metabolite observed after oral administration of TDF. However, the enhanced in vivo antiviral activity achieved after oral administration of TDF compared to intravenous administration of tenofovir itself suggests that the prodrug may deliver a certain portion of tenofovir directly into target cells.

Objectives. To develop a novel intracellular prodrug of tenofovir which is orally bioavailable, stable in plasma and selectively converted to tenofovir in target cells.

Results. A series of mono- and bis-phosphonamidate prodrugs of tenofovir were synthesized and characterized. The in vivo activity against HIV in tissue culture of several mono- and bis-amidates was at least 1000 fold higher than tenofovir. In addition to the tissue culture activity, stability in plasma versus MT-2 cell extracts was used to develop an SAR. Good stability in plasma and faster conversion to tenofovir in MT-2 cell extracts was achieved for both mono- and bis-amidates of tenofovir. The lead compounds selected for PK evaluation showed a good bioavailability of the prodrug in plasma and enhanced levels of tenofovir in PBMCs after oral administration of the prodrug in dogs.

Conclusions. Mono- and bis-phosphonamidates of tenofovir are new orally available prodrugs of tenofovir, which are stable in plasma but rapidly converted to tenofovir inside lymphatic cells.

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Alkylglyceryl and Alkoxyalkyl Esters of Antiviral Phosphonates

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Dept. of Medicine, VA Medical Center and Univ. of California, San Diego, La Jolla, CA 92093-0676

Nucleotide phosphonates such as cidofovir (CDV) and adefovir (ADV) are potent antivirals which are not well-absorbed orally due to the presence of the highly polar, negatively charged phosphonate moiety. The ability of phosphonates to cross biological membranes may also be substantially less than the corresponding nucleoside. We showed previously that esterification of acyclovir monophosphate with alkylglycerol or alkylpropanediol increases its oral bioavailability and effectiveness orally in woodchuck hepatitis. In this study, we report the synthesis and evaluation of a new series of phosphonate compounds obtained via esterification of cyclic CDV, CDV and ADV with long chain alkylglycerols and alkoxyalkanols. Cyclic CDV was alkylated with 1-bromo-3-hexadecyl-oxypropane to give 1-O-hexadecylpropanediol-3-cCDV (HDP-cCDV). The ring was opened with 0.5 N NaOH to afford 1-O-hexadecylpropanediol-3-CDV (HDP-CDV). The purified compounds were evaluated for antiviral activity in MRC-5 cells infected with human CMV (AD-169) and their 50% effective concentrations (EC_{50}) determined by DNA reduction (Hybriwix, Athens OH). The EC_{50} of CDV and cCDV was 0.46 μM versus 0.000002μM for HDP-CDV and 0.0003 μM for HDP-cCDV. Cytotoxicity was assessed and the selectivity indexes were calculated at 1×10^6 and 5×10^6 for the two compounds, respectively. Similar results were observed with an alkylglycerol adduct of CDV. Alkoxyalkanol analogs of CDV and cCDV were comparably active against HSV-1. The corresponding analog of ADV was >3 logs more active than ADVagainst HIV-1 infected cells. In conclusion, HDP-CDV and HDP-cCDV are highly active and selective against HCMV *in vitro*. Phosphonate compounds of this type appear to be worthy of further evaluation as antivirals for preclinical development.

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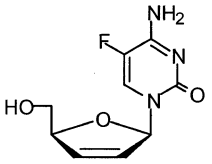
Fluorinated Nucleoside Analogs as Potential Antiviral Agents for the Treatment of Human Immunodeficiency Virus (HIV) and Hepatitis B (HBV) and C (HCV) Viruses. M. Hager and D. C. Liotta, Dept. of Chemistry, Emory University, Atlanta, GA; R. F. Schinazi, Emory University / VAMC, Atlanta, GA.

The inclusion of fluorine atoms in drugs oftentimes improves their pharmacological profiles relative to their non-fluorinated analogs. Specifically, fluorine substitution, *inter alia*, results in significant electronic changes with minimal steric perturbation, reduces the rate of oxidative metabolism and increases lipid solubility. We have devised a number of chemical approaches for preparing fluorinated and non-fluorinated nucleosides that have been designed to be active against HIV, HBV and HCV infections. Some of these compounds are shown below. Details of the chemical syntheses and biological evaluations of these compounds will be presented.



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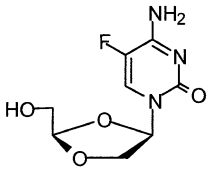
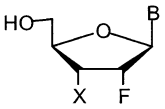


D-FDOC



X = H or OH;
B = purine or pyrimidine

Oral Session VI: Respiratory Virus, Other Infections

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A New Class of Capsid-Binder with Potent Inhibitory Activity Against Rhinovirus in Cell Culture.

K.G.Watson, W-Y.Wu, G.Y.Krippner, D.B.McConnell, B.Jin, P.C.Stanislawski, R.N.Brown, D.K.Chalmers, S.P.Tucker, R.Cameron-Smith, S.Hamilton, A.Luttick, J.Ryan, P.A.Reece

Biota Holdings, Melbourne, Victoria, Australia

Human rhinoviruses (HRV) have been implicated in approximately one-half of all cases of acute respiratory disease and no effective anti-rhinoviral agent is currently available for clinical use. X-ray crystallography has provided detailed structural information about rhinovirus and particularly the hydrophobic pocket beneath the canyon floor of the VP1 capsid protein. Over the past two decades various types of anti-viral capsid-binder have been discovered and several agents have been advanced into the clinic. We report here on the structure/activity relationships and molecular modeling studies for a new and large family of molecules which exhibit outstanding activity against human rhinoviruses in cell culture assays. Several of the new compounds show superior activity to known capsid-binders against a wide range of HRV strains. For example, when tested in parallel in a standard CPE assay, the lead compound BTA-188 inhibits HRV-14 with an EC_{50} of 1.0ng/ml compared with EC_{50} values of 30ng/ml and 3.2ng/ml for Pleconaril and Pirodavir respectively. BTA-188 thus represents an exciting new development candidate for the treatment of HRV disease.

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In Vitro Anti-Rhinovirus Spectrum and Potency of BTA188, a Novel, Oral Picornavirus Capsid Binder

FG Hayden[1], C Crump[1], PA Reece[2], K Watson[2], J Ryan[2], R Cameron-Smith[2] and SP Tucker[2] 1. University of Virginia School of Medicine, Charlottsville, USA. 2. Biota Holdings Limited, Melbourne, Aust.

Background: The picornavirus capsid has proven to be an excellent target for antiviral compounds, several of which have advanced to clinical rials. BTA188 is a new capsid-binding inhibitor, discovered through structure-based design (SBD), which exhibits high oral bioavailability in rodents and dogs and evidence of accumulation in nasal target tissue. We assessed its anti-human rhinovirus (HRV) spectrum and potency in cell culture in comparison to agents in clinical testing. **Methods:** Serial dilutions (0.001, 0.01, and 0.1 ug/ml) of BTA188 and the 3C protease inhibitor AG7088 and a fixed concentration of the capsid binder pleconaril at 1.0 ug/ml were tested in triplicate HeLa-I cell monolayers against 1) a panel of 100 numbered HRV serotypes and 2) 40 recent clinical HRV isolates. **Results:** BTA188 inhibited 87 of 100 numbered HRV serotypes (median EC50 = 0.01 ug/ml, range 0.0003 to >0.1 ug/ml)) and all 40 recent clinical isolates (median EC50 = 0.004 ug/ml, range <0.001 to 0.05 ug/ml) at <0.1 ug/ml. Pleconaril inhibited 81 of 100 numbered serotypes and 33 of 40 clinical isolates at 1.0 ug/ml. As expected, AG7088 inhibited all viruses tested (median EC50 = 0.012 μg/ml and 0.008 μg/ml, respectively). **Conclusions:** BTA188 has broad-spectrum, potent anti-rhinovirus activity in vitro. BTA188 and its analogs are attractive development candidates for the treatment and prevention of the common cold.

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Preclinical Pharmacokinetics of a New Rhinovirus Inhibitor, BTA188.

J Ryan[1], RW Sidwell[2], D Barnard[2], SP Tucker[1] , PA Reece[1]
[1]Biota Holdings Limited, Melbourne, Australia; [2]Institute. for Antiviral Research., Utah State Univ., Logan, UT, USA.

BTA188 is a capsid binding inhibitor with potent activity against a wide range of rhinovirus serotypes. A specific LC-MS assay was used to quantitate BTA188 concentrations in rat and dog plasma. In mice, anti-rhinoviral (type 2 human rhinovirus) activity as determined by *in vitro* assay in KB cells was used to quantitate serum BTA188 concentrations following oral gavage. Mean pharmacokinetic parameters for BTA188 following oral/iv doses to rat (60/10 mg/kg) and dog (10/1 mg/kg) are shown below:

	RAT (6M, 6F)		**DOG** (3M, 3F)	
	Males	Females	Males	Females
Half-life (h)	3.1	3.7	3.4	3.2
Cl (L/h/kg	0.56	0.43	1.2	1.1
V (L/kg)	2.5	2.3	5.8	5.2
Abs Bio (%)	62	64	21	28
T_{max} (h)	8.0	1.0	0.7	0.5

In toxicokinetic studies in rats, mean BTA188 plasma concentrations 24 hours post-dose (PD) ranged from 1 to 6 µg/ml over the dose range 60 to 180 mg/kg, and in dogs from 4 to 27 ng/mL over the dose range 3 to 20 mg/kg. Mean concentrations of BTA188 in the nasal epithelium of dogs 24 hours PD in a 28 day study were 25x plasma concentrations and exceeded the median EC_{90} for rhinovirus serotypes by factor of 3 at the no-effect dose. In mice, anti-rhinovirus activity equated to peak serum concentrations of BTA188 in excess of 50 µg/mL following a 33 mg/kg single oral dose. These data support the potential efficacy of oral BTA188 in human rhinovirus infections.

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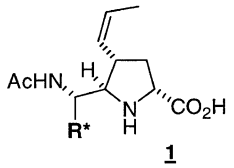
SAR Studies of Novel Pyrrolidine Influenza Neuraminidase Inhibitors: Identification of ABT-675 a Potent and Broad Spectrum Inhibitor.

C. Maring, K. McDaniel, A. Krueger, C. Zhao, M. Sun, D. Madigan, D. DeGoey, H.-J. Chen, M. C. Yeung, W. Flosi, D. Grampovnik, W. Kati, L. Klein, K. Stewart[1], V. Stoll[1], A. Saldivar, D. Montgomery, R. Carrick, K. Steffy, D. Kempf, A. Molla, W. Kohlbrenner, A. Kennedy, T. Herrin, Y. Xu, W. G. Laver[2]
Antiviral Research, Abbott Laboratories, Abbott Park, IL USA, [1]Structural Biology Research, Abbott Laboratories, and [2]Australian National University, Canberra, Australia

Inhibition of the viral enzyme influenza neuraminidase is an important approach for the treatment and prophylaxis of influenza infection. The design of inhibitors for neuraminidase has been aided by the ready availability of x-ray crystal structures detailing the structural features of the five sub-sites (S1-5) of the enzyme active site. Using an iterative structure based method, we have discovered and optimized inhibitors that exploit a significant and previously unrecognized hydrophobic interaction in the putative "amine binding" sub-site 2 (S2) of neuraminidase. Our investigations have focused on a series of five member ring pyrrolidine inhibitors that led to the optimized core structure **1** for S(1-3). Subsequent studies detailing the nature and scope of the **R*** group directed toward S4-5 have produced potent inhibitors both in vitro and in cell culture assays. The structure activity relationships and crystal structure data that have been developed for the identification of ABT-675 a potent inhibitor of influenza neuraminidase will be presented.



1

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R170591, a New Antiviral with picoMolar Activity against Respiratory Syncytial Virus. K. Andries, M. Moeremans, T. Gevers, R. Willebrords, S. Sommen, J. Lacrampe*, F. Janssens Janssen Research Foundation, Beerse, Belgium and *Val De Reuil, France

A cell-based assay (using MTT as endpoint) was used to identify compounds that are able to inhibit fusion of HeLa cells, infected with RSV. Synthesis of >300 analogues of a lead compound led to the selection of R170591 (MW 395). The in vitro antiviral potency of this benzimidazole derivative (IC50=150 picoM) exceeds that of ribavirin almost 100,000 times (IC50=10 microM). Antiviral activity was confirmed for human (subgroup A and B) and bovine RSV, and in human respiratory tract cell lines. Efficacy in cytopathic effect inhibition assays correlated well with efficacy in virus yield reduction assays. A concentration of 10 nanoM reduced RSV production 1000-fold in multi-cycle experiments, using a low or a high multiplicity of infection. Time of addition studies pointed to a dual mode of action: inhibition of virus-cell fusion early in the infection cycle and inhibition of cell-cell fusion at the end of the replication cycle. Two resistant mutants were raised and shown to have single point mutations in the F-gene (S398L and D486N). Pretreatment of cotton rats by inhalation resulted in >90 % reductions of both lung and BALF RSV titers, as assessed by a newly developed quantitative RT-PCR assay.

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Cellcept (the prodrug of mycophenolic acid) is active against RSV disease in mice

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[1.] Roche Discovery, Welwyn, Hertfordshire, UK [2.] Department of Microbiology and Immunology, University of Newcastle upon Tyne, UK

Respiratory syncytial virus (RSV) is the single most common cause of viral bronchiolitis during infancy. The only recognised treatment (ribavirin) is suboptimal due to its topical mode of delivery and dose-limiting toxicity. RSV disease has an immunopathological basis. It is unknown whether ribavirin acts through its weak antiviral activity or by inhibition of inosine monophosphate dehydrogenase (IMPDH) resulting in reduced T and B lymphocyte activity. Mycophenolic acid (MPA) is more potent than ribavirin both as an antiviral against RSV and as an inhibitor of IMPDH. It can be delivered orally and safely as Cellcept. A model of RSV-induced pneumonia has been established in Balb/c mice using an intranasal challenge of high titre (10^7pfu) RSV A2 strain. Oral administration of Cellcept at 100mg/kg daily from days 1 to 6 post-infection significantly reduced ($p<0.001$) severity of disease by day 7 as measured by weight loss (23.2% in untreated controls compared to 8% in Cellcept treated). A positive therapeutic benefit was still observed when treatment indication was delayed until day 5 post-infection. The positive effect of Cellcept on weight loss was reflected in the lung histology of the mice. These preliminary data suggest Cellcept should be further examined as a potential treatment for RSV disease in children.

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Sensitivity of Clinical Virus Isolates from Two Pleconaril Viral Meningitis Studies. D.C. Pevear, T.M. Tull, M.S. Collett and M.A. Menegus*, ViroPharma Incorporated, Exton, Pa, and *University of Rochester Medical School, Rochester, NY, USA

Enteroviruses (EVs) are the most common virus infection of the central nervous system. Pleconaril is a novel small molecule inhibitor of human picornavirus replication that is being developed for the treatment of diseases associated with these viral infections. Here we investigated the drug sensitivity of clinical virus isolates obtained from throat-swab cultures from patients enrolled in two viral meningitis trials during 1997 and 1998. Of 95 subjects (65 on drug (D) and 30 on placebo (P)) that were EV positive on pre-dosing study day 1, 44 subjects (24 D and 20 P) had virus positive throat swab cultures at some time after dosing (study days 2-7). Paired pre- and post-dosing virus positive samples from these 44 subjects were tested for drug sensitivity in a blinded fashion. Post-dosing samples from three subjects possessed virus populations that were at least 10-fold less sensitive to pleconaril than pre-dosing samples in a viral cytopathic effect assay. The etiologic agents of infection in these patients were determined to be echovirus type 30 (EV30, two patients) and echovirus type 9 (EV9, one patient). The two EV30 isolates became insensitive to pleconaril (IC_{50} changes from 0.001 and 0.002 μM respectively to >10 μM) while the EV9 isolate remained sensitive to pleconaril (IC_{50} change from 0.007 μM to 0.097 μM). No virus was isolated by throat swab culture from any of the pleconaril-treated subjects on study day 8. The three post-dosing virus samples with altered drug sensitivity each consisted of a mixture of both wild type and variant virus. Upon cell culture passage in the absence of pleconaril, wild type virus overtook the cultures in all three cases, indicating that the variant viruses were substantially less fit than the respective wild type viruses. Cloned EV9 variants were found to be extremely thermal-labile. Molecular cloning and sequencing of viral capsid protein VP1 identified the identical amino acid change in the EV30 variants (Ile to Met at VP1 amino acid 96). Based on alignments with coxsackievirus B3 for which an x-ray crystal structure has been determined, this residue is predicted to be in the EV30 drug-binding pocket. The EV9 variant had two amino changes, both of which were located outside of the drug-binding pocket. The results indicate that pleconaril-insensitive variant viruses can be isolated at a low frequency (2 of 65; 3%) from drug-treated subjects, but that such viruses appear less fit than their wild type counterparts.

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Modoc Virus Encephalitis in SCID Mice and Hamsters, a Model for the Study of Strategies for the Treatment of Flavivirus Encephalitis

J. Neyts,[1] J. Paeshuyse,[1] P. Leyssen,[1] A. Van Lommel,[2] & E. De Clercq[1]
[1]Rega Institute for Medical Research, K.U.Leuven, B-3000 Leuven, Belgium; [2]Division of Histopathology, K.U.Leuven, B-3000 Leuven, Belgium

Many flaviviruses are known to cause severe encephalitis in man (e.g. West Nile encephalitis; Japanese encephalitis, Saint Louis encephalitis, Murray Valley encephalitis, tick-borne encephalitis, Russian spring-summer encephalitis). We employed the murine Modoc virus (MODV) to establish a convenient model of Flavivirus encephalitis in small laboratory animals that can be used to study strategies for the treatment of flavivirus encephalitis. Intraperitoneal inoculation of severe combined immunodeficiency (SCID) mice or immunocompetent hamsters with MODV results in severe encephalitis and death. Brains of hamsters with signs of encephalitis show massive infiltration of the *bulbus olfactorius* and *cerebral cortex* with peripheral lymphocytes. Perivascular cuffing is observed in small and medium sized blood vessels. The formation of endematoes necrotic foci, in which the normal tissue structure has been completely destroyed, is noted. Deeply eosinophilic, degenerating neurons in, and surrounding, these affected areas appear to be involved in neuronophagia and the formation of glial nodules. Spongiosis of the molecular layer of the cerebellum is observed and numerous Purkinje cells are involved. Ultrastructural analysis of infected neurons shows extensive proliferation and dilatation of vesicular structures in which virus-like particles are observed. By means of *in situ* hybridisation with a MODV specific riboprobe and immunohistochemistry with a MODV specific polyclonal antiserum, viral replication is detected in structures such as the *bulbus olfactorius*, *cerebral cortex*, *thalamus*, *hippocampus*, molecular and Purkinje layer of the *cerebellum* and *medulla oblongata*. Viral RNA and antigens are detected in the cytoplasm of infected neurons. Interestingly, hamsters that survive MODV encephalitis show neurological sequelae as has been described for patients that recover from Japanese encephalitis. The Modoc/SCID and Modoc/hamster model may prove instrumental for the development of new strategies for the treatment of infections with these viruses.

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Treatment of aerosolized cowpox virus infection in mice with aerosolized cidofovir. M Bray[1], M Martinez[2], C Roy[3], [1]Virology, [2]Pathology, [3]Aerobiology Div, US Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD.

The threats of smallpox as a potential bioterrorist weapon and of monkeypox as a naturally occurring disease require the continuing evaluation of antiviral medications that could block orthopoxvirus infection, mitigate illness, reduce secondary transmission and prevent death. We have used a model of lethal respiratory tract infection with aerosolized cowpox virus in weanling BALB/c mice to test the efficacy of antiviral therapy of virulent orthopoxvirus infections. We previously found that mice can be successfully treated with one subcutaneous (s.c.) injection of the DNA polymerase inhibitor cidofovir (HPMPC, Vistide®). In an attempt to further enhance the effect of treatment, we tested the efficacy of delivering cidofovir by small-particle (1 micron) aqueous aerosol to the same respiratory tract tissues infected by the aerosolized virus. We found that a single aerosol treatment, before or after viral challenge, was more beneficial than a larger s.c. dose at the same time point. Mice that received approximately 25 mg/kg of cidofovir by aerosol, either the day before or two hours after infection, remained active, continued to gain weight, and showed a minimal increase in lung weight on day 8 postinfection (a marker of broncho-pneumonia), compared to placebo-treated controls. Aerosol treatment two hours after infection also prevented the development of significant pulmonary bronchiolar lesions. By contrast, mice injected s.c. with 100 mg/kg of cidofovir at the same time point had lower body weights and increased lung weights, developed bronchiolar lesions, and had 10-fold higher lung viral titers on day 8 than the aerosol-treated mice. Our data suggest that aerosolized cidofovir would be effective for the prophylaxis and early postexposure therapy of human infection resulting from orthopoxvirus aerosols.

Poster Session III: Hepadnavirus, Respiratory Infections

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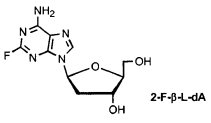
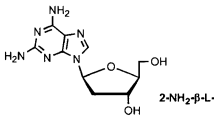
2-Fluoro- and 2-amino- β-L-2'-deoxyadenosine: Stereospecific syntheses and antiviral activities

D Dukhan,[1] M Bryant,[2] J-P Sommadossi,[2] J-L Imbach[1] and G Gosselin[1].
[1]Laboratoire Coopératif Novirio-CNRS-Université Montpellier II, France; [2]Novirio Pharmaceuticals, Cambridge, USA.

It was only at the beginning of the 1990's that unnatural L-nucleosides were considered as potential antiviral agents and evaluated as such.[1] We have recently discovered that the previously described β-L-2'-deoxy-adenosine (β-L-dA) exhibits potent activity against human hepatitis B virus without concomitant cytotoxicity.[2]



2-F-β-L-dA



2-NH$_2$-β-L-dA

In this work, we will report a structure-activity relationship study on the effect of modification on the adenine 2-position on antiviral activity. A fluorine atom and an amino group have been introduced to produce 2-fluoro-2'-deoxyadenosine (2-F-β-L-dA) and 2-amino-2'-deoxyadenosine (2-NH_2-β-L-dA), respectively. The stereospecific syntheses and data from biological evaluations will be presented.

1) Wang, P.; Hong, J. H.; Cooperwood, J. S.; Chu, C. K. *Antiviral Res.* **1998**, *40*, 19-44.
2) Dukhan, D.; Pierra, C.; Bryant, M.; Sommadossi, J.; Imbach, J.-L.; Gosselin, G. *Antiviral Res.* **2000**, *46*, A62.

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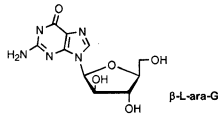
L-Enantiomer of 9-(β-arabinofuranosyl)guanine: Stereospecific synthesis and biological evaluations

D Dukhan,[1] M Bryant,[2] J-P Sommadossi,[2] J-L Imbach[1] and G Gosselin[1]

[1] Laboratoire Coopératif Novirio-CNRS-Université Montpellier II, France; [2] Novirio Pharmaceuticals, Cambridge, USA.

During recent years there has been a growing interest in unnatural L-enantiomer nucleosides as antiviral agents[1]. However, purine β-L-arabinofuranosyl nucleosides have received less attention and to the best of our knowledge, 9-(β-L-arabinofuranosyl)guanine is hitherto unknown . Since the D counterpart (β-D-Ara-G) possessed important biological activities[2,3], we carried out a stereospecific synthesis of β-L-araG in order to evaluate its potential antiviral activities.



β-L-ara-G

The stereospecific synthesis and data on biological evaluations of β-L-ara-G will be presented.

1) Wang, P.; Hong, J. H.; Cooperwood, J. S.; Chu, C. K. *Antiviral Res.* **1998**, *40*, 19-44.
2) Kisor, D.; Plunkett, W.; Kurtzberg, J.; Mitchell, B.; Hodge, J.; Ernst, T.; Keating, M.; Gandhi, V. *J Clin Oncol* **2000**, *18*, 995-1003.
3) Rodriguez, C. O.; Gandhi, V. *Cancer Res.* **1999**, *59*, 4937-43.

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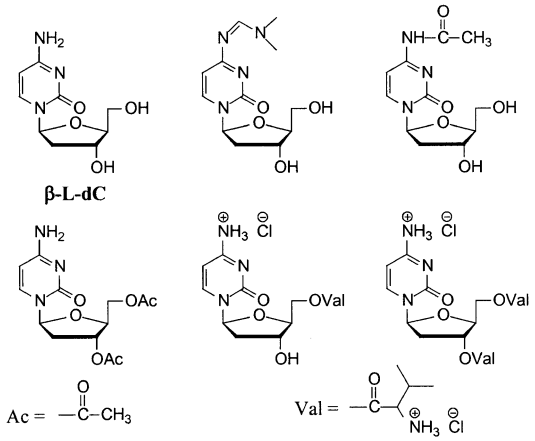
Synthesis of potential prodrugs of β-L-dC, a potent and selective anti-HBV agent.

S Benzaria,[1] C Pierra,[1] J-L Imbach,[1] M Bryant,[2] J-P Sommadossi,[2] and G Gosselin.[1]
[1]Laboratoire Coopératif Novirio-CNRS-Université Montpellier II France; [2]Novirio Pharmaceuticals, Cambridge, USA.

β-L-2'-Deoxycytidine (β-L-dC) was recently shown to be a potent, selective and specific anti-HBV agent, both *in vitro* and *in vivo*. Low oral bioavailability (16.4%) prompted the development of prodrugs with more favorable oral absorption profiles. Several β-L-dC derivatives have been synthesized.



The syntheses of the potential β-L-dC prodrugs displayed in the above figure will be described, and preliminary stability studies will be reported. Further studies of physicochemical characteristics and pharmacokinetic parameters are reported in abstracts by Pierra, et al., and Cretton-Scott, et al., respectively.

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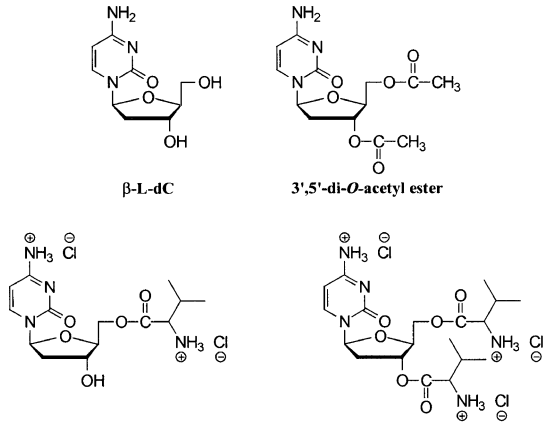
Comparative studies of selected potential prodrugs of β-L-dC, a potent and selective anti-HBV agent.

C Pierra,[1] S Benzaria,[1] G Gosselin,[1] J-L Imbach,[1] M Bryant,[2] and J-P Sommadossi[2].
[1]Laboratoire Coopératif Novirio-CNRS-Université Montpellier II, France; [2]Novirio Pharmaceuticals, Cambridge, USA.

Several potential prodrugs of the anti-HBV agent β-L-dC have been prepared with the aim to increase the oral bioavailability of the parent compound. Three compounds (see Figure) were selected and their physicochemical properties (including stability, solubility and logP value) and pharmacokinetic parameters were determined (see also Cretton-Scott, et al.).



Based on physicochemical characteristics, ease of synthesis and oral bioavailability, we concluded that the 3',5'-di-*O*-valinyl ester was the most promising candidate for further development.

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An Automated High-Throughput Assay for the Identification of Chemical Entities Effective Against Hepatitis B Virus.

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The pharmaceutical industry has attempted to maximize its ability to screen drug candidates by the use of automation and improved detection methods. Cell-based assays for identification of viral inhibitors are complex and difficult to automate. In particular, hepatitis B virus (HBV) screens traditionally have been labor-intensive due to the length of time required in tissue culture to achieve the desired antiviral effect and the complexity of the techniques required for detection of HBV DNA. We developed an automated tissue culture system and a sensitive, automated microtiter plate hybridization method, which replaces PCRs and dot blots, to produce a high-throughput assay for screening chemical libraries for antiviral activity against HBV. Stable transfected human hepatocytes (2.2.15 cells) are seeded into 96-well microtiter plates containing compounds of interest. The media is replenished every three to four days with fresh compound, and after nine days the cell supernatants are collected and lysed for use in a hybridization detection assay specific for HBV DNA. The non-radioactive hybridization assay utilizes an ELISA format for ease of automation. In this approach, PCR fragments of the HBV genome of approximately 500 base pairs in length that have been immobilized onto a 96-well plate are simultaneously hybridized with the lysed cell supernatants and digoxigenin labeled detection probes. The captured hybrids are then detected through the use of a peroxidase-conjugated anti-digoxigenin antibody and a chemiluminescent substrate. Both the tissue culture and the hybridization assays have been automated through systems incorporating Tecan Genesis workstations customized with various 96-well compatible components, including an automated tissue culture incubator. This methodology provides a primary screening capacity of over 6,000 compounds (84 microtiter plates) in a two-week period. Details of the methods and a complete description of the automation will be presented.

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Development of plasma assays for the enantioseparation of L-nucleosides in plasma by high performance capillary electrophoresis (HPCE).

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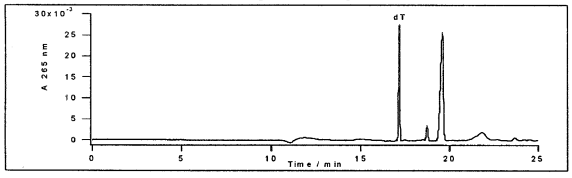
Unnatural L-deoxynucleosides have been shown to have potent antiviral activity against hepatitis B virus infection *in vitro* and in animal models. Clinical trials are underway to determine the efficacy of these compounds in man. Highly sensitive assays for the accurate measurement of these compounds are needed to aid with the pharmacokinetic evaluation in biological fluids. Assays for the measurement of L-deoxynucleosides over a wide range of concentrations in plasma were developed using HPCE. Spiked plasma (500 µl) was extracted using a solid phase extraction process followed by HPCE. To increase sensitivity, stacking of large volume of sample introduced into the capillary was obtained during electrophoresis. In order to achieve both sensitivity and enantioseparation, several parameters were investigated to aid in the sample stacking process, including ionic strength, pH, SDS, organic solvent, and additives. L-deoxythymidine was completely separated from plasma components with limit of quantitation (LOQ) at 65 ng/ml (RSD<3%) using UV detection. L-deoxycytidine was completely resolved from D-deoxycytidine using cyclodextrin as chiral separation agent.



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Antiviral Activity of Adefovir Dipivoxil in Transgenic Mice Expressing Hepatitis B Virus. J. D. Morrey, J. G. Julander, and R. W. Sidwell. Institute for Antiviral Research, Utah State University, Logan, Utah, USA.

Adefovir dipivoxil (bis-POM PMEA) (100 mg/kg/day, bid, per os, 10 days) was evaluated in transgenic mice expressing hepatitis B virus (Guidotti, et al., J. Virol. 69:6158-6169). Liver HBV DNA, as determined by Southern blot hybridization, was reduced from a mean of 3.0 pg of viral DNA per μg of total DNA in placebo-treated mice to below detectable limits at <0.1 pg/μg. The HBV DNA in the serum was reduced >80-fold in the Adefovir-treated mice as compared to the placebo-treated mice. Some of the serum DNA titers were below the limits of detection (3,000 genome equivalents/mL serum). Treatment of 3TC at a very high concentration (300 or 500 mg/kg/day), using the same treatment schedule as Adefovir, reduced serum HBV DNA by only 4-fold. During the course of the experiment, serum viral titers were remarkably reduced by 10- to 1,000-fold in the untreated mice, which may have been due to the effects of the pre-bleed procedure or other stresses such as oral gavage. The reduction of virus titers in control animals may have masked the extent of viral reduction from Adefovir dipivoxil treatment, such that the antiviral effects of the drug may have been greater than actually measured. These studies demonstrated that Adefovir dipivoxil will be an adequate positive control for future experiments, and that virus reduction in control animals need to be delineated or eliminated to provide the best antiviral studies.
(Supported by Contract N01-AI-05404 from Enteric & Hepatic Diseases, NIH, NIAID, DMID, EHDB)

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Combination of Adefovir and DNA Vaccine in the Duck Hepatitis B Virus Infection Model

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Antiviral therapy of chronic HBV infection faces the problem of eliminating viral CCC DNA from infected hepatocytes. The aim of our study was to determine in the DHBV infection model whether adefovir treatment, may suppress viral CCC DNA in infected hepatocytes and whether a combination with naked DNA immunization may enhance the curing of infected hepatocytes. First, we examined the antiviral effect of adefovir in primary fetal duck hepatocyte cultures. Drug administration after virus inoculation induced a dose-dependent inhibition of viral DNA synthesis and reduction but not elimination of CCC DNA. Moreover, adefovir treatment starting before virus inoculation and maintained for 6 to 9 more days did not prevent the initial formation of CCC DNA in primary hepatocytes. *In vivo*, administration of adefovir prior to the virus inoculation delayed the onset of viremia, but did not prevent DHBV infection to occur. Then, we evaluated *in vivo* the efficacy of a strategy combining adefovir administration to inhibit viral replication, and DNA based immunization with a pCI preS/S plasmid expressing the DHBV large envelope protein to enhance the antiviral immune response. The experiment included chronically infected ducks distributed as follows: i.p. administration of adefovir (9 animals), intramuscular DNA immunization with pCI preS/S plasmid (9 animals), combination with adefovir and pCI preS/S plasmid (9 animals). Controls included 8 untreated ducks, and 9 birds which received empty pCI plasmid. A profound inhibition of viremia levels was observed in animals treated with adefovir with or without DNA vaccination, and was maintained during the treatment period. After drug withdrawal, the rebound of viral replication was initially similar in the 2 groups. After the 3rd boost with a pre-S/S plasmid, a more pronounced decrease in viral replication in the bitherapy group was observed compared with the adefovir monotherapy or DNA immunization groups. Our results suggest that i) adefovir exhibits a profound antiviral activity in infected hepatocytes, ii) a combination with a specific DNA immunization approach may induce a sustained decrease in viral replication.

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***In Vivo* Antihepadnaviral Activities of Combination of Famciclovir, Lamivudine, and Foscarnet**

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Hepadnaviruses infection can lead to liver disease, which may evolve to cirrhosis and hepatocellular carcinoma. One of the important strategies in hepatittis B virus (HBV) therapy is the use of multidrug combination to control HBV infection. This present study was carried out to evaluate the benefits of 10 days' short-term chemotherapy of lamivudine (3TC, at dose of 4mg/kg/day) combined with famciclovir (FCV, at dose of 8mg/kg/day) and/or foscarnet (PFA, at dose of 62.5mg/kg/day) in the duck hepatitis B virus (DHBV) system *in vivo*. One-day-old ducklings infected by DHBV were used as experimental model to observe anti-HBV effect of the combination chemotherapy. The levels of viremia, the total intrahepatic DHBV load and CCC DNA were measured by dot blot hybridization and southern blot hybridization. Viral DNA was also measured by *in situ* hybridization. The results showed that the effects of tri-combination chemotherapy were more significant than that of bi-combination chemotherapy using any two of them or that of the individual components on inhibition of DHBV-DNA during drug treatment. The inhibitive rate in serum of the tri-combination, bi-combination and monodrug chemotherapy were 99.13% (3TC and FCV plus PFA), 49.85% (3TC plus PFA),88.52% (3TC plus FCV), 96.57% (PFA plus FCV),64.50% (3TC),48,08% (PFA),93.33% (FCV) respectively while the total viral DNA load of tir-combination in the liver was reduced by 77.66% compared to the control level. During the follow-up period all parameters of active virological replication returned to those for the age-matched controls. Further investigation of the antiviral activities of different drug combinations is therefore required.

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Hepatitis B Virion (HBV) Clearance is Extremely Rapid in a Subset of Chronically Infected Individuals. SR Lewin[1], T Walters[2], S Bowden[2], G Lau[3], R. Ribeiro[4], A Perelson[4], S Locarnini[2]. [1]Victorian Infectious Diseases Service and [2]Victorian Infectious Diseases Reference Laboratory, The Royal Melbourne Hospital, Parkville, Victoria, Australia; [3]Department of Medicine, Queen Mary Hospital, Hong Kong People's Republic of China; [4]Los Alamos National Laboratory, Los Alamos, NM.

BACKGROUND: Following treatment of hepatitis B virus (HBV) infection with antiviral therapy, a biphasic response in HBV plasma DNA has been previously described. A true estimate of the half-life of HBV virions and infected cells has been limited by the currently available quantitative assays for HBV. **METHODS:** Using a novel real-time PCR and molecular beacon based assay to quantify plasma HBV DNA, we studied 13 HBV-chronically infected individuals treated with either lamivudine (3TC; n=4) 150 mg/day or 3TC 150 mg/day plus famciclovir (FCV; n=9) 500 mg tds for 12 weeks. Serial ALT and serum HBV DNA were determined at week 0,1,2,4,6,8,10 and 12. **RESULTS:** The real-time PCR based assay was linear over a wide dynamic range, with accurate detection ranging from 100 to 10^9 copies per ml. The assay had an interassay variation of 0.3 log and a strong correlation at high (>10^6 copies per ml) and low (<10^6 copies per ml) viral loads with the Digene Hybridisation assay and Roche Monitor assay respectively (r=0.9; p<0.0001). Following antiviral therapy, a biphasic response was seen in most individuals with two distinct patterns. The first pattern demonstrated a rapid first phase (RFP) decline in viral load (n=7; mean $t_{1/2}$=0.58±0.13 days) followed by a plateau in the second phase (mean $t_{1/2}$=34 days (range 2.5->700 days). The second pattern demonstrated a slower first phase (SFP) decline (n=6; mean $t_{1/2}$=1.87±0.43 days), followed by a distinct second phase decline (mean $t_{1/2}$=10 days (range 7->700 days). A RFP response was seen in 5/9 individuals taking FMC/LMV combination therapy and 2/4 taking LMV. However, there was a larger delay before the effect of therapy was observed in the case of LMV alone (median 3.02 vs. 1.14 days, p=0.06). **CONCLUSIONS:** Clearance of HBV viral particles is more rapid in a subset of individuals than previously reported. Rapid clearance is not associated with the specific drug regimen.

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Cross-Resistance Analysis of Lamivudine, Adefovir, and Entecavir Using a Cell Culture Model of HBV Replication W. E. Delaney IV[1], C. E. Westland[1], H. L. Yang[1], K. Y. Lin[1], K. Das[2], E. Arnold[2], A. K. Jain[1], M. D. Miller[1], C. S. Gibbs[1], and X. Xiong[1]. [1]Gilead Sciences, Foster City, CA, USA. [2]CABM Rutgers University, Piscataway, NJ, USA.

Clinical use of lamivudine and famciclovir for the treatment of chronic HBV frequently results in the selection of drug-resistant strains of virus. In order to treat patients with liver disease who have lamivudine-resistant HBV and to facilitate the development of combination regimens that can prevent the emergence of drug resistance, there is a need to identify compounds with activity against both wild-type and drug-resistant HBV strains. Adefovir (PMEA) and entecavir (BMS 200,475) are nucleotide and nucleoside analogs, respectively, that have demonstrated potent activity against wild-type HBV and are now in phase III clinical trials. Using a model based on transient transfection of HepG2 cells we assayed the ability of lamivudine, adefovir, and entecavir to inhibit the replication of wild-type HBV and HBV containing the L528M, M552I, and L528M + M552V polymerase mutations (mutations observed frequently in patients failing famciclovir or lamivudine therapy). Our results confirm that the M552I and L528M + M552V mutations confer a high degree of resistance to lamivudine. Entecavir demonstrated high potency against wild-type HBV (IC_{50} of 0.002 μM) in agreement with previous reports. Entecavir was effective against HBV encoding L528M, a famciclovir resistance mutation that is also proposed to compensate for the reduced replication rates of M552 mutants. However, the lamivudine resistance mutations M552I and L528M + M552V increased the IC_{50} of entecavir approximately 860 and 180 fold, respectively. In contrast, adefovir retained efficacy against all of the drug-resistant mutants we tested (preliminary data indicates IC_{50} values within 3 fold of wild-type). A model of the 3D-structure of HBV polymerase based on the crystal structure of HIV RT suggests that the β-methyl branched sidechains of V and I at position M552 contribute to the resistance of HBV polymerase to lamivudine and entecavir, but would not interfere with adefovir binding. In summary, these data suggest that HBV mutants selected by lamivudine administration are cross-resistant to entecavir but not adefovir.

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Development of a Cell based assay system for hepatitis C Virus infection

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The development of human liver cell lines capable of supporting HCV propagation is critical to understanding virus replication and pathogenesis, as well as for evaluating anti-HCV compounds. Attempts to establish an in vitro system in which HCV infection and replication could be followed were reported. However, these efforts did not result in a practical and reproducible system, that could be used as a functional biological assay. To develop a cell-based assay, in which cells could be infected *in vitro* with HCV, sera from HCV-infected patients were used to infect cell lines, such as MOLT-4, Hep-G2 and FLC-4. Sera from normal donors (anti-HCV antibodies negative) were used as negative controls in the infection experiments. HCV-RNA was tested in supernatants after one day and after six days in culture. The cells were split weekly, and HCV-RNA was determined in cell pellets by RT-PCR followed by dot blot hybridization. HCV-RNA could be detected in the cells during three weeks in culture. After each split, the presence of HCV-RNA in supernatant could be detected only after six days in culture and not after one day, indicating accumulation of HCV-RNA with time. HCV infection of the cells was further confirmed by in-cell RT-PCR in situ hybridization. Viral replication was evident from the presence of (-)strand RNA detected in the cultured cells which could be maintained for at least 21 days. The existence of HCV infectious particles in the cell cultures is supported by preliminary data showing the ability to use supernatants to infect new cells. Feasibility of using this cell based system for evaluating efficacy of anti-viral agents is being tested using human monoclonal antibodies to the envelope proteins of the hepatitis C virus.

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Toxicological evaluation of the anti-HBV agent β-L-deoxythymidine.

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[1]Novirio Pharmaceuticals Cambridge, USA; [2]Laboratoire Coopératif Novirio-CNRS-Université Montpellier II, France.

β-L-Deoxythymidine (L-dT) is an unsubstituted, unmodified β-L-deoxynucleoside with potent antiviral activity against hepadnaviruses *in vitro* and *in vivo*. It has high oral bioavailability and a favorable PK profile in rodents and non-human primates.

The safety of L-dT was evaluated in rats (Sprague-Dawley) and cynomologus monkeys (*Macaca fascicularis*) following acute exposure and repeated dosing for 4 weeks by oral administration. No overt signs of toxicity were observed in either rats or monkeys during acute or 28-day studies at dosages up to 2000 mg/kg/day. There were no L-dT-related effects in either rats or monkeys on body weights, food consumption, clinical pathology parameters (hematology and serum chemistry), organ weights (absolute and relative), or histopathology. No macroscopic lesions attributable to L-dT were observed at necropsy, nor were there any microscopic findings considered to be related to L-dT. Based on the results of this study, the no observed adverse event level (NOAEL) for L-dT following acute dosing or repeated dosing for 28 days by oral gavage in rats and monkeys was 2000 mg/kg.

L-dT was tested for mutagenic activity and acute and subacute toxicity. There was no evidence of mutagenicity in the *Salmonella* or *E. coli* plate incorporation mutation assay with L-dT concentrations up to 5000 μg/plate. L-dT was not clastogenic *in vitro* in Chinese hamster ovary (CHO) cell chromosome aberration assays at concentrations up to 5000 μg/mL or *in vivo* to male or female mice at doses up to 2000 mg/kg. The favorable safety profile of L-dT *in vivo* supports its further development for the treatment of chronic HBV infection.

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Development Of A Screening Program To Evaluate The Efficacy, Range, And Mechanism Of Action Of Anti-HCV Compounds.

M Wenzel Mathers, M Huang, LA Pallansch, and RW Buckheit, Jr., Southern Research Institute, Frederick, MD, USA

In the United States, about 4 million people are infected with hepatitis C virus (HCV). HCV infection usually results in chronic hepatitis among whom 10 to 20% will progress to life-threatening cirrhosis and another 1 to 5% will develop a liver cancer. Development of an effective therapy will eradicate infection early in the course of disease and thereby prevent progression to end stage disease. Due to the lack of an effective HCV cell culture system it has been difficult to evaluate potential anti-HCV drugs. Our laboratory has designed a screening program to help identify candidate HCV drugs using both cell based surrogate model assays, as well as with the development of molecular target based assays. Our lab has adapted and optimized cell-based assays using Bovine Diarrhea Virus (BVDV) to initially determine the efficacy of potential HCV compounds. BVDV is a member of the same Flavivirus family as HCV, and shares similar genetic structures and functions. The primary screening assay is a cytopathic effects based assay and has been modified for use as a high throughput robotics based assay system. Combination antiviral assays, multiplicity of infection assays, and plaque reduction assays are performed using this surrogate model as well. Molecular target-based assays have been developed to help determine the mechanism of action of potential anti-HCV compounds. These assays include the NS2/3 protease assay, the NS5B polymerase assay, and the IRES assay. The effect of antiviral compounds on viral RNA production can also be quantified using PCR-based Taqman technology. Combining the above mentioned assays provides a useful strategy for the preclinical evaluation of potential anti-HCV compounds. By identifying promising new compounds this program can be useful in the development of new drugs for patients infected with the HCV virus.

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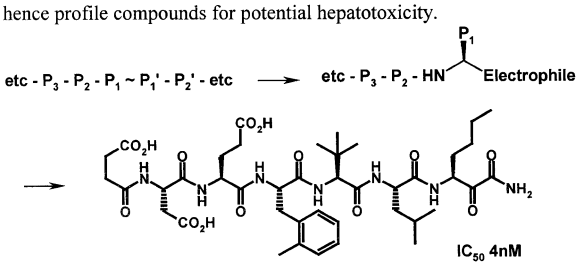
Design and Synthesis of Hepatitis C NS3 Proteinase Inhibitors

P.S. Jones, D. Allsop, M.R. Attwood, R. Beevers, J.M. Bennett , R. Bradford, A. Bryson, A.D. Campbell, A.M. Campbell, M.G. Carr, E. Conway, R.M. Dunsdon, M. Fulleylove, S. Gilbert, D.N. Hurst, J.R. Greening, B.K. Handa, S. Harris, P.C. Howroyd, N.S. Jennings, W. R. Jiang, S. Jordan, P.B. Kay, E. Keech, S.T. Kingston, J. King-Underwood, R. Lazell, M.A. O'Brien, A. Ochert, T.M. Raynham, K.P. Stenson, B. Sutton, C.S. Wilkinson, T.C.I. Wilkinson, F.X. Wilson.

Roche Discovery Welwyn, Welwyn Garden City, Herts, UK.

Hepatitis C Virus (HCV) is the cause of the majority of cases of transfusion-associated hepatitis and a significant proportion of community-acquired hepatitis worldwide. Infection by HCV can lead to a range of clinical conditions including an asymptomatic carrier state, severe chronic active hepatitis, cirrhosis and, in some cases, hepatocellular carcinoma. Current estimates show ~300 million chronically infected worldwide.

Starting from the observation that the virus possesses a chymotrypsin-like serine proteinase, we have designed potent electrophile-based inhibitors in three structural classes. The design and synthesis of these is detailed, together with the use of rat hepatocyte cultures to correlate *in-vitro* observations with *in-vivo* effects on the liver and hence profile compounds for potential hepatotoxicity.



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Novel 3-(4-Pyridyl)indole Inhibitors of the Hepatitis C Virus NS3 Serine Protease.

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An estimated 1% of the global population is infected by hepatitis C virus (HCV). Chronic infection invariably leads to cirrhosis, hepatocellular carcinoma and liver failure in later life. The only approved therapies are interferon α alone or in combination with ribavirin (Rebetol). However, these therapies suffer from providing poor sustained response rates and frequent side-effects. The development of new therapies to effectively treat HCV infection is therefore of paramount importance. The NS3 serine protease of HCV is a widely studied target for the development of novel anti-HCV agents. We have developed an *in vitro* coupled transcription and translation system in rabbit reticulocytes to generate a ^{35}S methionine labelled NS5A/5B polypeptide, which is efficiently processed upon incubation with the NS3 protease domain. We have used this system to screen for potential protease inhibitors, and have identified novel 3-(4-pyridyl)indole inhibitors active in this system. The rapid optimisation of this series using array synthesis, and the differing activity of these inhibitors in the transcription and translation system compared with a classical biochemical system employing a 5A/5B peptide substrate, in which the compounds are apparently inactive, are discussed.

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Analysis of the mechanisms of iterferon alpha-induced clearance of HCV replication by gene expression profiling

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Interferon-α (IFN-α) mono-therapy has been widely used for the treatment of chronic HCV infection. The HCV subgenomic replicon system of Lohmann *et al* (1) provides us with a tool to study the mechanism(s) of IFN-α clearance of HCV from Huh7 cells. We have shown previously that IFN-α 2a (Roferon) clears the replicon in this system at an IC50 of 1 IU/ml (2). Multiple RNA extracts were prepared from HCV replicon containing cell lines and control cell lines +/- IFN-α 2a (1000 IU/ml) for 24hrs and used to generate biotinylated cRNA. These were hybridised to Hu6800 Affymetrix gene array chips. The expression pattern of selected genes was also determined by kinetic time-course PCR. The results show that the transcription of multiple genes are significantly modulated in response to IFN-α treatment in these cells. A number of these modulated transcripts were derived from genes regarded as classic 'interferon-stimulated genes' while others may represent genes whose association with IFN-α was previously unknown. A comparison of IFN-induced genes in the presence and absence of the HCV replicon indicates that some transcripts are counter modulated by HCV replication and hence may represent mechanisms by which HCV evades the IFN-induced antiviral response.

(1) Lohmann V., Korner F., Koch J., et al (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285 (5424), 110-113.

(2) Najera, I., Laxton, C., Granycome, C. et al (2000) Characterisation of a HCV subgenomic replicon with reduced susceptibility to interferon alpha-2a. 7th International Hepatitis C and related viruses meeting, Golden Coast, Australia, 3-7th Dec 2000.

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Use of the HCV subgenomic replicon for the selection and characterisation of variants with reduced susceptibility to interferon alfa-2a

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Alpha interferon is the only current treatment for HCV infection, either alone or in combination with ribavirin. However, rates of sustained virological response are less than 20% with alpha interferon monotherapy and around 40% in combination with ribavirin. One suggested pathway of alpha interferon action is through the induction of double-stranded RNA-activated protein kinase (PKR) which leads to the inhibition of protein synthesis via phosphorylation of eIF2α. Several studies have suggested a viral mechanism for the reduced susceptibility to the drug, including the non-structural protein 5A (NS5A) and the structural protein E2.

We investigated the effect of interferon alfa-2a (IFN) on the replication of the HCV subgenomic replicon in Huh7 cells (Lohman *et al.* Science 1999; 285: 110-113). IFN inhibited replicon replication and showed an IC50 (inhibitory concentration at which the replicon RNA is reduced by 50%) of 1 IU/ml. As the replicon contains non-HCV sequences, such as the EMCV IRES and neo resistance gene, we eliminated these as the targets of IFN action by generating an HCV monocistronic replicon with a hygromycin resistance gene. The copy number of this monocistronic replicon also was reduced, highlighting the sensitivity of the HCV replicon to IFN action.

In order to clarify the role of viral proteins in HCV resistance to alpha interferon, an HCV replicon-containing cell line resistant to IFN was generated by subsequent *in vitro* passages in the presence of increasing concentrations of the drug. After 21 passages a cell line resistant to IFN was selected, showing a shift in the IC50 of greater than 100-fold.

To investigate whether this effect was due to the selection of IFN resistant variants or to a cellular effect, RNA from the cells was isolated and introduced into naïve Huh-7 cells. After selection with G418 in the absence of IFN, new cell lines with reduced susceptibility to IFN were isolated. Results obtained with the newly isolated cell lines will be presented.

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Identification of host pathways required for HCV replication using the HCV subgenomic replicon

K.L. Hilyard[1], E.J. Murray[1], S-Y. Chang[3], H.A. Stirnadel[2] and M. Paterson[2]
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The HCV subgenomic replicon system of Lohmann *et al* has been used to identify host pathways required for HCV RNA replication. Multiple cellular RNA extracts were prepared from HCV repli-con-containing cell lines and control cell lines and used to generate biotinylated cRNA probes which were hybridised to Hu6800 Affymetrix gene array chips. Kinetic time-course PCR was also used to confirm mRNA transcripts that were differentially expressed between these two groups of samples. The results presented illustrate the inherent experimental variability seen at several different levels: (i) at the level of chip batch-to-batch, (ii) experiment-to-experiment and (iii) cell line-to-cell line. A comparison of the mRNA transcripts detected in a replicon positive and control cell lines found that approximately 11% of the expressed genes on the Hu6800 Affymetrix chip were significantly differentially expressed between replicon and control. However, a pair-wise analysis of expression profiles from several different replicon-containing cell clones and control lines found that this number varied greatly between different comparisons. As a result, we chose to select transcripts that were only modulated in all of the pair-wise comparisons generated. Using this approach, we identified 41 transcripts. The data presented here illustrate the value of this cell replication system for the identification of host pathways required for HCV replication and hence the identification of novel host drug targets for HCV therapy. We have also shown the inherent variability both in the gene array techniques and the in vitro replication model that require careful experimental design to overcome.

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) *Science* 285(5424): 110-113.

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Evaluation of human monoclonal antibodies to HCV in the HCV-Trimera model: a mouse model for HCV infection

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There are immediate needs for effective therapies for preventing and managing HCV infection. Therapeutic human antibodies to HCV that are able to reduce HCV viral load could serve either as a stand-alone or as an adjunct treatment in patients with chronic infection. Development of neutralizing antibodies to HCV was hampered by the lack of in vitro systems as well as suitable animal models to screen for effective antibodies. We have developed the HCV-Trimera mouse model, in which human liver fragments infected with HCV are transplanted into immunosuppressed mice. Viremia is followed by quantitation of HCV-RNA in sera of these mice. The presence of (-) strand HCV-RNA confirms viral replication in engrafted liver. This model has been used to evaluate the ability of anti-HCV human monoclonal antibodies to reduce viral load and to inhibit HCV infection. Using peripheral B cells isolated from individuals infected with HCV genotype 1b, human monoclonal antibodies (HMAbs) to the HCV envelope protein (E2), were generated and characterized *in vitro*. Three HMAbs, HCV-AB 68, CBH-2 and CBH-5, were further tested in the HCV-Trimera mouse model. The antibodies were effective in inhibiting HCV infection of human liver fragments as measured by reductions in mean viral load and the percentage of HCV positive animals as compared to control groups. Furthermore, administration of HMAb HCV-AB 68 to HCV-Trimera mice with established viremia resulted in significant reduction of the mean viral load as well as reduction of the percentage of positive animals suggesting a possible role in treatment. Thus, the HCV-Trimera mouse model offers a powerful tool for simulating human HCV infection and for evaluating therapeutic antibodies. Our results indicate the feasibility of using HMAbs to HCV for prevention and treatment of HCV infection.

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Sequence specific cleavage of the 3'-UTR of HEV RNA mediated by Di- and Mono-hammerhead ribozymes targeted against GUX sites to inhibit viral replication

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Hepatitis E virus (HEV) is the major cause of enterically transmitted non-A, non-B hepatitis and is responsible for significant morbidity and mortality, particularly in developing countries. It accounts for 30-50% of acute sporadic viral hepatitis both in children and adults. Mortality due to HEV is 1-2% and in pregnant woman the rate is 10-20%. Ribozymes are catalytic molecules that can be designed to cleave specific RNA sequences. To investigate the potential use of synthetic ribozymes for the treatment of HEV infection, we designed and synthesized hammerhead ribozymes targeting conserved 3' untranslated region (UTR) of HEV RNA. This region is cis-acting regulatory element which plays an important role in viral replication. Therefore cleavage of this region by hammerhead ribozymes may be a good way to inhibit viral replication and cure the liver disease caused by HEV. We designed Di-hammerhead ribozyme (two catalytic motifs in tandem) targeted against GUA and GUU placed 13 nucleotides apart and a Mono-hammerhead ribozyme against GUU in the 3'-UTR of HEV RNA. These ribozymes were chemically synthesized, cloned in pcDNA-3 and sequenced. Cleavage efficiency of these ribozymes were tested *in-vitro* which is Mg^{++} ion dependent. Around 80% of cleavage was observed under optimal conditions. Cleavage was observed to increase with increase in $MgCl_2$ concentration and time of incubation at 37^0 C upto 2 hrs. Significant cleavage was also observed at simulated physiological conditions. The disabled ribozymes did not cleave the target RNA, indicating cleavage was due to active ribozyme. These are the first ribozymes reported against HEV RNA that are found to potentially cleave 3'-UTR *in-vitro*. These *in-vitro* studies suggest the efficiency of these ribozymes can be tested *in-vivo* and potential application of these trans-acting hammerhead ribozymes can be used for gene therapy of liver diseases caused by HEV.

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ABT-675 Dissociates More Slowly than Oseltamivir from Both A and B Strains of Influenza Neuraminidase

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The apparent association and dissociation rate constants for the inhibition of B/Memphis/3/89 and A/Tokyo/3/67 influenza neuraminidases were measured by monitoring the approach to reach equilibrium using the progress curve method. The dissociation rate constant for ABT-675 from B/Memphis neuraminidase was 1.9×10^{-5} s^{-1}, a value which was 18-fold slower than the rate constant measured for oseltamivir. The corresponding half-times for dissociation calculate to be about 10 hours for ABT-675 compared to 33 minutes for oseltamivir. The significance of the slow dissociation rate constant results is that, under conditions when circulating drug has been removed from the site of viral infection, ABT-675 should still produce a continued and greatly prolonged inhibition of neuraminidase activity relative to oseltamivir. The association rate constants were fast enough so that both drugs should bind to neuraminidase within seconds when present at concentrations above 0.1 μg/ml. Similar studies demonstrated an 18-fold slower dissociation rate constant for ABT-675 relative to oseltamivir against A/Tokyo/3/67 neuraminidase. Thus, ABT-675 dissociates much more slowly than oseltamivir from both A and B strains of influenza neuraminidase.

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Analogues of the Influenza Neuraminidase Inhibitor GS 4071: Isosteric Substitutions of the Carboxylic Acid Group.

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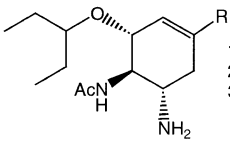
Tamiflu (oseltamivir phosphate, GS 4104) **1** is an orally administered influenza neuraminidase inhibitor recently approved for the treatment of influenza infection. The ethyl ester prodrug **1** is readily hydrolyzed *in vivo* to afford the active species GS 4071 (**2**). GS 4071 is a potent inhibitor of both influenza A and B neuraminidases (typically, IC_{50} = 0.7 nM and 4.1 nM respectively). X-ray crystallographic analysis of GS 4071 bound in the neuraminidase enzyme active site revealed the carboxylic acid group makes critical interactions with the guanidino moiety of three arginine residues. In order to more fully understand these interactions, a series of analogues with carboxyl group isosteres (**3**, R = phosphonate, sulfonyl carboxamide, tetrazole) was available. From this series, analogue **3** (R = phosphonate) was selected for further study as a potent inhibitor of influenza A and B with IC_{50} values of 1.7 nM and 100 nM respectively. Further analysis was performed by X-ray crystallography to elucidate binding interactions of the phosphonate group in the neuraminidase active site.



1 (R = CO_2Et, H_3PO_4 salt)
2 (R = CO_2H)
3 (R = $PO(OH)_2$, $CONHSO_2R$, tetrazole)

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***In vitro* and *in vivo* susceptibility to the anti-influenza neuraminidase inhibitor zanamivir, in comparison with amantadine.**

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The absence of reports to date describing the emergence of resistance to zanamivir (a novel neuraminidase inhibitor) in clinical trials, may be an indication of a lesser potential for the emergence of clinically important resistance, as compared to the influenza A virus M2 ion channel inhibitor, amantadine. However, there have been no reports directly comparing the potential for development of resistance to both drugs. In the present study, the potential for emergence of resistance to both drugs, in influenza A virus infections, was compared both *in vitro* and *in vivo*. Following passage of influenza virus in MDCK cells, in the presence either zanamivir or amantadine, variants with reduced susceptibility were isolated after 9 and 2 passages, respectively. The sensitivities of these variants to zanamivir and amantadine were 122 and >92 times less than those of the wild-type virus, respectively. The *in vivo* susceptibility to both drugs was also examined by serial passage of the virus isolated from mice treated with zanamivir (0.4 mg/kg, bid, in) or amantadine (2 mg/kg, bid, po). In these experiments a 50-fold reduction in the susceptibility was found only in amantadine treated animals, following 5 serial passages. Furthermore, when virus infected mice were repeatedly administered with either high doses of zanamivir (15 mg/kg, bid, in) or amantadine (75 mg/kg, bid, po), mutant virus was only isolated from mice treated with amantadine. These mutant viruses, selected within 5 days after starting drug administration, were subsequently found to have amino acid substitutions in the M2 protein. The present studies indicate a reduced potential for emergence of influenza A virus with reduced susceptibility to zanamivir, as compared to amantadine, confirming an important advance of zanamivir as an anti-influenza medication.

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***In vitro* efficacy of combinations of influenza virus replication inhibitors.**

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Therapeutic use of antiviral agents could lead to the rapid emergence of drug resistant variants which have been associated with the transmission of resistant virus strains from treated patients to close contacts. The generation of drug resistant variants also contributes to the failure of single drug therapies. To assess the possible interaction among influenza replication inhibitors, dual combinations of neuraminidase inhibitors and anti-influenza drugs were tested against influenza A virus replication in MDCK cells in a cytoprotection assay. We have also evaluated the drug related toxicity when used in combination. Beforehand, the individual 50% inhibitory (IC_{50}) and 50% toxicity (TC_{50}) concentration for each of the compounds had been determined by using the cytoprotection assay. Dual combinations of drugs showed an additive effect on virus replication inhibition. Such studies would provide insights into whether drug combinations yield enhanced therapeutic effect compared to single drug treatment. This could lead to development of efficient chemotherapy as well as better understanding of the mode of action of each drug. Studies are also underway to determine the effect of drug combinations on viruses resistant to neuraminidase inhibitors.

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Phenotypic and Genotypic Studies of Influenza Virus Isolates from NAI30008, a Clinical Efficacy Study of Relenza in the Treatment of Subjects with Asthma/COPD. D Gor, F Mirzai, AC Candlin, MS Walters & SM Tisdale. Dept. of Immunology and Virology, GlaxoWellcome R&D, Stevenage, UK

Patients with underlying conditions such as asthma or chronic obstructive pulmonary disease (COPD) may experience a prolonged and severe course of disease, and are at increased risk of complications following influenza infections. RelenzaTM (zanamivir), a novel compound that inhibits the neuraminidase (NA) enzyme of influenza A and B viruses, is licensed in several countries for the treatment of influenza A and B in adults and children. To date, no isolates resistant to RelenzaTM have been identified from clinical trials and surveillance studies of RelenzaTM in immunocompetent individuals. A virology sub-study was performed to monitor the drug susceptibility of viruses obtained from a double blind randomised placebo-controlled study designed to evaluate the safety and efficacy of RelenzaTM in the treatment of subjects with asthma/COPD (NAI30008). Virus culture in MDCK cells was attempted from 256 throat swabs taken at Day 1 (baseline), 2, 3 and 6 from both RelenzaTM- and placebo-recipients. This yielded 118 isolates (60 from RelenzaTM and 58 placebo-treated subjects) with a titre sufficient for susceptibility testing by the neuraminidase inhibition assay. The IC50 ranged from 0.28 -35nM, with no shift in susceptibility between matched isolates. The isolate with the highest IC50 (35nM) was from a subject whose symptoms resolved within 2.5 days. The Day 6 sample from this subject was culture negative. Two of the 131 Day 6 samples tested were culture positive, although only one of these had a titre high enough for the NA assay, the other isolate was identified as positive by PCR. The IC50 for the Day 6 isolate was 0.6nM, with a baseline of 0.56nM. Sequence analysis of the full NA gene and HA1 portion of the haemagglutinin gene was also performed on isolates characterised by the NA inhibition assay and on all culture fluids from Day 6 samples. There was no evidence of mutations that might give rise to a reduced susceptibility to RelenzaTM. Consistent with previous treatment and prophylaxis studies, susceptibility monitoring of influenza isolates from this study in asthma/COPD subjects has shown no evidence for RelenzaTM resistance.

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The Detection Of Influenza Virus Resistant To Oseltamivir Carboxylate Requires Serial Sampling Of Nasal Swabs.

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Oseltamivir carboxylate (GS4071, the active metabolite of oseltamivir) is a potent inhibitor of influenza A & B neuraminidases, effective in the treatment and prevention of influenza. For the amantadinamine class of anti-influenza drugs, approximately 30% of treated patients carry resistant virus, often emerging within 48 hours of starting treatment. *In vitro* and *in vivo* studies indicate that resistance to the neuraminidase inhibitors will occur less readily. However, it is important to accurately quantify this incidence and characterise any resistant genotypes. Neuraminidase with reduced sensitivity to oseltamivir carboxylate has been detected at low incidence (4/418) in an adult treatment trial with oseltamivir. In this trial, nose and throat swab samples were taken for virological examination on study days 1, 2, 4, 6 and 8. The day 1 (pre-treatment) and last culture positive samples from each patient were tested in a sensitive and reproducible neuraminidase phenotypic assay. Resistant virus was detected transiently on either days 4 or 6 of the study and was observed in the patient's last culture positive sample only. In 2/4 cases, virus carrying mutant neuraminidase was found to be in the presence of excess wild-type virus. In contrast, another clinical trial of oseltamivir treatment in naturally acquired influenza infection in adolescents and adults, swab samples were taken only at pre-treatment (day 1) and study day 3. There was no evidence for the emergence of drug resistant virus for any of the treated subjects (0/554). The emergence of resistance to oseltamivir is a low frequency event that occurs transiently late in treatment. Resistance has only been detected by taking frequent sequential samples from subjects to identify the last culture positive sample. Sampling virus at a fixed, mid-treatment time point recorded a zero incidence of resistance. These observations suggest that the timing of sample collection is critical for the detection of resistance to neuraminidase inhibitors as a class.

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An epidemiological model of influenza to investigate the potential transmission of drug resistant virus during community use of antiviral treatment for influenza.

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An epidemic model of influenza has been constructed and used to predict the transmission of wild type and drug resistant influenza virus during community use of antiviral treatments and/or chemoprophylaxis for influenza. Data derived from clinical studies with oseltamivir have been used to investigate the potential community impact arising from the emergence of drug resistant virus strains during treatment with neuraminidase inhibitors (NAIs). **Methods:** A deterministic compartmental epidemic model was constructed including five population groups: susceptible, post-contact, infected aysmptomatic, infected symptomatic and immune. A heterogeneous age structure was used to include age related variables for virus attack rate, population mixing, viral shedding and immunity. Model validation utilised published scientific data and specific information from clinical trials with oseltamivir. Conservative values, likely to over-estimate spread of viral resistance, were assigned to parameters where exact estimates were not available. **Results:** This model reproduces characteristics of typical influenza epidemic outbreaks for a population with characteristics of the USA. The rate of spread of drug resistant virus was simulated for a wide range of treatment scenarios encompassing current and maximum realistic future levels of symptomatic treatment and chemoprophylaxis. This analysis predicts that incidence of resistant virus in the NAI treated population, will remain at the rate of de novo incidence among treated individuals. No significant secondary transmission of resistant virus is predicted. **Conclusions:** The key factor limiting transmission of viruses currently identified with NAI resistance mutations, is their low epidemiological fitness (transmissibility), which we infer from experimental data to be <10% of that of wild-type virus. NAI resistant virus would only be predicted to spread through secondary transmission if its relative fitness were > 90%. However, there is a much higher rate of de novo emergence of resistant virus following treatment with amantadines than with NAIs. This, together with data from animal studies suggesting higher relative fitness of amantidine resistant viruses, is shown to predict amantadine resistant viruses to have a higher probability of spread in the community than NAI resistant viruses, under the assumption that transmissibility correlates with in vivo viral fitness.

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Influenza Virus Carrying an R292K Mutation in the Neuraminidase Gene is not Transmitted in Ferrets

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The neuraminidase (NA) inhibitors provide a new therapeutic option for the treatment and prevention of influenza. *In vitro* and *in vivo* studies indicate that the incidence of resistance to this class of drugs will be much lower than that seen with the amantadinamines. Influenza A virus with an R292K mutation in the NA gene has been selected *in vitro* by serial passage with zanamavir, oseltamivir carboxylate and RWJ270201, with reduced sensitivity to all 3 drugs. It is therefore important to assess the potential for the transmission of this resistant virus should it emerge in the treated patient population. Previous studies have shown that viruses carrying the R292K NA mutation have markedly reduced infectivity in both mice and ferrets. This would indicate a reduced potential for transmission. We have studied the transmission in ferrets of a clinical isolate of influenza A/N2 carrying the NA R292K mutation compared with the corresponding wild-type virus from the same subject. Donor ferrets (n=4 per group) were inoculated intranasally with mutant or wild-type virus and each housed with 3 naïve contact ferrets. All 4 donor ferrets inoculated with wild-type virus were productively infected and transmitted virus to all 12 contacts, who in turn had high viral titres in their nasal washes. In contrast, 2 of the donor ferrets inoculated with mutant virus were productively infected. For 1 of these donors there was evidence of virus transmission to 1 of the 3 contacts. A positive, low titre was detected in the nasal wash from this ferret on 1 day only. The other donor ferret transmitted virus to all 3 contacts producing high viral titres in nasal washes over several days. The mutant virus in this donor had partially reverted to wild-type which had transmitted to the contacts. These data confirm the lower infectivity of influenza virus carrying an R292K NA mutation in ferrets and shows that transmission of resistant virus will be unlikely in widespread treatment of influenza with NA inhibitors.

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The Analysis of Structure – Antiinfluenza Activity Relationships on the Basis Molecular Lattice Model for Macrocyclic Pyridinophanes and their Analogs

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In this research we tried to solve QSAR task for some macrocyclic pyridinophanes (MCP) and their analogs in framework molecular lattice model. Such approach provides a generation of homogeneous system of structural parameters, which reflect local, integral and field characteristics of molecular structure for effective solution of diverse "structure-property" tasks. The earlier this approach was applied successfully for MCP anticancer activity analysis and prediction. In this work the antiinfluenza activity was represented in rank scale and trend-vector procedure was used for constructing of QSAR models. The researched set consisted of 37 compounds. Statistic characteristics for QSAR models in this case are satisfactory (R=0,90-0,93; CVR=0,76-0,86). It is shown that electronegative substituents in pyridine cycle and/or availability of nitrogen atom in β position to pyridine cycle promote to antiviral activity. Partial atom charges were determined which influence on activity. The active sites of potential receptor were determined in region of molecular cavity. The "wall" of potential receptor was detected at a periphery of molecule. On base of our QSAR investigations it is shown the possibility for drug design of high active antivirals. This work was partially supported by the INTAS foundation (grant INTAS 97-*31528)*.

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Synthesis and antiviral properties of 2,6 - and 3,5 - disubstituted pyridines and their analogs

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Our previous investigations have revealed antiviral activity of proteolysis inhibitors E-aminocaproic acid (E-ACA) and para-aminomethyllbenzoic acid (PAMBA). 2,6- disubstituted pyridines and their analogs were synthesized and their antiviral activities were analyzed in this study. Molecules of these compounds consist of "nucleus" (Py or Ar) and two symmetrical fragments: E-ACA-carbonyl or PAMBA-carbonyl. Antiinfluenza (anti-Flu) activity of the compounds in dose 10^{-3}M was studied *in vitro* on the model of A/Hong Kong/1/68 (H3N2) reproduction in tissue cultures of chorioallantoic membranes of 12-14 days old chick's embryos. Antiherpetic (anti-HSV) and antiadenovirus activities of the compounds in doses 10^{-4}M were studied on models of reproduction of HSV-1 and Ad-5 in cell culture Hep-2. E-ACA and PAMBA have not antiviral activity in these doses. PAMBA-aminocarbonyl contains compounds with Py or Ar in "nucleus" of molecules showed anti-Flu action that was not dependent from nature of this "nucleus" compounds with Py but not Ar in "nucleus" of molecules and contained PAMBA-carbonyl or E-ACA-carbonyl fragments demonstrated anti-HSV activity (65% and 42% of reduction of count of infected cell accordingly) also 2,6-dimethyl-3,5-disubstituted pyridines were synthesized. 3,5-dihydrazine-carbonyl-2,6-dimethylpyridine showed high anti-HSV (62%) and low anti-Ad activity. Schiff bases 3,5-bis (anthracene – 9-formyl-hydrazinecarbonyl)-2,6-dimethylpyridine demonstrated high anti-Ad (63%) activity and it did not showed anti-HSV action. Both compounds had not anti-Flu activity. The results of research demonstrate us that synthesis and antiviral properties studying of 2,6 - and 3,5 - disubstituted pyridines and their analogs is very perspective field of research. This research was supported in part by INTAS Grant 97-31528 and all the authors are indebted to INTAS Foundation courtesy.

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Respiratory Syncytial Virus (RSV) of a Chinese Medicinal Herb -- Antiviral Agent No.1 in Hela Cell Culture Li Hong-yuan, Yie Hong, Liu Dian-li, Zhou Kun, Tong Kui-Ming. Department of Epidemiology, School of Public Health, Harbin Medical University, Harbin 150001, China

Antiviral Agent No.1 is an aqueous extract from a Chinese medicinal herb. To observe the anti-RSV activities of Antiviral Agent No.1, the anti-RSV activities of Antiviral Agent No.1 were observed by means of the technique of Hela cell culture using Ribavirin as a positive control. Antiviral Agent No.1 is found to be a potent inhibitor of RSV replication in Hela cells when the agent was added 2 hours post infection, with a 50% toxic concentration (TC_{50}) of 14.87mg/ml, a 50% inhibitory concentration (IC_{50}) of 0.42 mg/ml and a therapeutic index (TI, TC_{50}/ IC_{50}) of 35; while Ribavirin with TC_{50} 25.44 μ g/ml, IC_{50} 0.92 μ g/ml and TI 28 respectively. There exits a significant correlation between the logarithmic concentration and inhibition ratio of cytopathic effect in both of these two agents ($P<0.005$). The results indicate that Antiviral Agent No.1 as well as Ribavirin can inhibit RSV in vitro obviously. An active compound of anti-RSV has been purified by a chemical method from the Chinese medicinal herb.

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Anti-influenza Virus Activity of a Chinese Medicinal Herb -- Antiviral Agent No.1 in MDCK Culture Li Hong-Yuan, Yang Yi-Shu Liu Mi-Feng, Liu Dian-li, Zhou Kun, Tong Kui-Ming. Department of Epidemiology, School of Public Health, Harbin Medical University, Harbin 150001, China

Antiviral Agent No.1 is an aqueous extract from a Chinese medicinal herb. To observe the Anti-influenza Virus activities of Antiviral Agent No.1, the Anti-influenza A/Wuhan/359/95(H3N2) and B/Shenzhen/12/97 activities of Antiviral Agent No.1 were observed by means of the technique of MDCK cell cultures using Ribavirin as a positive control. Antiviral Agent No.1 is found to be a potent inhibitor of A/Wuhan/359/95(H3N2)replication in MDCK Culture when the agents were added 2 hours post infection, with a 50% toxic concentration (TC_{50}) of 60.53mg/ml, a 50% inhibitory concentration (IC_{50}) of 5.14mg/ml and a therapeutic index (TI, TC_{50}/ IC_{50}) of 11.78; while Ribavirin with TC_{50} of 13.20 μ g/ml, IC_{50} of 2.09 μ g/ml and TI of 6.32 respectively. Antiviral Agent No.1 is also found to be a potent inhibitor of influenza B/Shenzhen/12/97replication in in MDCK Culture when the agent was added 2 hours post infection, with a 50% toxic concentration (TC_{50}) of 60.53mg/ml, a 50% inhibitory concentration (IC_{50}) of 8.88mg/ml and a therapeutic index (TI, TC_{50}/ IC_{50}) of 6.81; while Ribavirin with TC_{50} of 13.20 μ g/ml, IC_{50} of 1.58 μ g/ml and TI of 8.34 respectively. There exits a significant correlation between the logarithmic concentrations and inhibition ratios of cytopathic effects in both of these two agents ($P<0.005$) for influeza A/Wuhan/359/95(H3N2) and B/Shenzhen/12/97. The results indicate that Antiviral Agent No.1 as well as Ribavirin can inhibit influenza replications in vitro obviously.

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Characterization of Soluble Human Parainfluenza Virus Type 1 Hemagglutinin-Neuraminidase Glycoprotein Expressed in Insect Cells

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Human parainfluenza virus types 1 (hPIV-1), 2, and 3 represent significant respiratory pathogens for which no antiviral treatment is currently available. To characterize the biochemical functions of hPIV-1 hemagglutinin-neuraminidase (HN) glycoprotein, a potential target for antiviral therapy, we cloned and expressed a soluble portion of hPIV1 HN (amino acid residues 137-575), lacking the hydrophobic membrane anchorage region, in insect cells using the baculovirus secretion expression system (pMelBac, Invitrogen). The expressed HN protein was purified through cation-exchange chromatography followed by metal affinity chromatography, using the 6xHis epitope introduced at the carboxyl-terminus of the recombinant protein. N-terminal amino acid sequence analysis of purified HN indicated that the honeybee melittin secretion signal peptide was correctly removed during post-translational processing. Further characterization revealed that the purified HN protein was N-glycosylated and exhibited neuraminidase activity whose characteristics resembled those of the native HN protein of hPIV-1 virions. The establishment of this expression and purification system has allowed us to further explore the biochemical characteristics of paramyxovirus HN and to obtain material that could be suitable for X-ray crystallography studies.

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Biochemical Study of the Antiviral Activity of R170591, a Potent Inhibitor of Respiratory Syncytial Virus Replication.

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BACKGROUND: Cell based assays identified R170591 as an extremely potent (IC50=150 picoM) and very selective (CC50=100 microM) inhibitor of RSV induced cytopathic effects [K. Andries et al., this meeting]. METHODS/RESULTS: The virus was preincubated with R170591 (10, 1, 0.1 and 0.01 μM), with ribavirin (50, 10 and 2 μM), or with solvent. HeLaM cells were then inoculated with virus at high m.o.i. SDS-electrophoresis of total cell extracts, pulse labelled with 35S, demonstrated inhibition of the synthesis of two RSV induced proteins at all R170591 concentrations. These results were confirmed in that the viral fusion protein could not be detected by Western blotting. With ribavirin viral protein synthesis was blocked only at 50 μM. To assess if R170591 could affect RNA expression (either directly or indirectly), viral RNA was analysed with RT-PCR. Although not quantitative, the results indicated that viral RNA synthesis was essentially completely blocked at 1 μM and 100 nM, with >90% reduction still apparent at 10 nM. With ribavirin viral RNA synthesis was partially blocked only at 50 μM. CONCLUSIONS: These data confirmed the antiviral activity of R170591 and implicated an early event (i.e. RNA expression or earlier) in the viral replication cycle as target. In addition these data confirmed the superior potency of R170591 as compared with ribavirin.

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Quantitative Determination of RSV RNA Copy Number in Cotton Rat Lung Tissue and BALF.

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An RT-PCR assay was developed for the quantification of RSV RNA in lung tissue homogenates and BALF. The method uses newly designed primers in the nucleocapsid coding region, N gene, and an internal homologous standard (IS). The IS was designed by replacing a probe recognition sequence with a heterogeneous sequence allowing differential hybridisation-ELISA. A dilution series of RNA from a cell culture derived viral stock was used to generate a standard curve.
The coefficient of correlation, obtained by linear regression analysis of the data plotted on a log-log scale, was consistently >0.95. A 4 $\log_{10}$ dynamic range was obtained by analysing different dilutions of the amplicons in the ELISA. The lower limit of detection was 10^2 pfu per g specimen. This sensitivity could be further increased tenfold by increasing the number of PCR cycles. The median intra-assay reproducibility was 0.13 $\log_{10}$ for values ranging from 3.4 to 5.9 $\log_{10}$ arbitrary pfu/ml. Independent assays indicated that 0.5 $\log_{10}$ differences could be consistently reproduced. The average ratio of the number of arbitrary RNA copies per pfu, calculated from 8 standard curves, was 276 ± 105. Titers of BALF samples determined with plaque titration and RT-PCR were strongly correlated.

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In Vitro Activity Of BTA 188: A Novel, Picornavirus Capsid Binding Inhibitor With Potent Anti-Rhinovirus Activity.

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Picornaviruses (PV) are responsible for significant human disease and include human rhinoviruses (RV), the primary cause of the common cold. To date no antiviral drugs for PV infections have been approved, although some are in clinical trials. Therefore, two classes of capsid binding compounds were developed to inhibit PV, especially RV. Of the compounds tested, BTA188 was the most efficacious against RV in both cytopathic effect (CPE) reduction and neutral red assays. For the 45 RV serotypes tested, EC_{50} values ranged from 0.1 ng/ml (RV40) to 2000 ng/ml (RV-25). BTA188 inhibited 89% of the RV serotypes tested with EC_{50} values ≤ 90 ng/ml (mean = 9 ng/ml, median = 3.2 ng/ml) by CPE assay. Using the same ≤ 90 ng/ml criterion, pirodavir inhibited 80% of the RV serotypes (mean = 20 ng/ml, median = 10 ng/ml) and pleconaril inhibited 69% of the RV serotypes (mean = 16 ng/ml, median = 10 ng/ml) tested. The compound also inhibited three 1997 RV clinical isolates with EC_{50} values ranging from10-1000 ng/ml. In a virus yield reduction assay, BTA 188 potently inhibited RV-2 with an EC_{90} = 0.73ng/ml. BTA 188 was evaluated for efficacy using multiplicities of infection (MOI) from 0.025-15. Striking inhibition of RV-2 replication occurred even at MOI = 15 (EC_{50} = 3.2 ng/ml). Time of addition studies showed that the compound optimally inhibited RV-2 when added at 2 h post virus exposure, although significant inhibition was observed when added as late as 8 h after virus exposure (EC_{50} = 20 ng/ml). The compound was not virucidal for RV-2. In actively growing KB cells, the 50% cytotoxic concentration (IC_{50}) = 2000 ng/ml and in stationary KB cells, the IC_{50} = 10,000 ng/ml. The compound also inhibited enteroviruses (EV) including echoviruses (EC_{50} = 200-9000 ng/ml), polioviruses (EC_{50} = 200-3,000 ng/ml), and coxsackie A and B viruses (EC_{50} = 590-10,000 ng/ml). These data suggest that BTA 188 warrants further investigation as a potentially useful agent for treating RV infections and selected EV infections.

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On the Antagonistic Combined Effect of Ribavirin and Some Picornavirus Replication Inhibitors

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The combined effect of ribavirin and a number of picornavirus replication inhibitors with known mechanisms of action have been tested on the replication of poliovirus type 1 (Mahoney). Combining ribavirin in dual combinations with enviroxime, disoxaril, arildon, S-7 (methylthiopyrimidine), guanidine hydrochloride, PTU-23 (N-phenyl-N'-3-hydroxyphenilthiourea) and HBB resulted 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; mopyridone] a specific togavirus replication inhibitor, has been tested on the growth of SFV (also a plus-RNA genome, but capped on its 5'-end). That combination manifested a synergistic antiviral effect. The observed synergy could be explained by the attack on different targets in the replicative cycle of SFV. Two principle differences could be noticed in poliovirus model as compared to the SFV: (i) ribavirin cannot exert one of the components of its tri-factorial antiviral mechanism (lack of a cap-structure in poliovirus genome); (ii) the antiviral effect of ribavirin, wholly cell-mediated, is diminished (significant host cell protein and RNA shut-off). Besides, it could be suggested that every compound that inhibits picornavirus replication "limits" poliovirus targets for ribavirin effects. These reasons could substantiate the observed diminished antiviral effect of ribavirin when combined with other picornavirus replication inhibitors.

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A Sensitive and Precise Method for Detection and Quantification of Human Rhinovirus

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Human rhinoviruses (HRV), the major etiological agents of the common cold, are highly divergent viruses that include more than 100 different viral serotypes. This antigenic and sequence diversity has hindered efforts to produce a sensitive and robust method of detection and quantification of HRV in laboratory and clinical settings. We have developed a viral RNA purification method and two-step RT-PCR assay, based on TaqMan™ chemistry, which is able to quantify a broad range of virus serotypes. The PCR amplifies conserved sequences within the 5' UTR of the viral genomic RNA and is able to detect 8 HRV 5' UTR cDNA copies per PCR. For most HRV serotypes, this limit of detection is equivalent to or better than 0.25 plaque forming units (PFU) per ml. The limit of quantification is 50 copies of cDNA per PCR. The inter-assay precision of the purification and RT-PCR is better than two-fold, indicating good assay precision. In addition, we have used this assay to determine that HRV serotypes contain different numbers of RNA genomes per PFU, reflecting a possible difference in in vitro infectivity among serotypes. The range of serotypes detected, the sensitivity and the quantitative range are all significant improvements over current methods. These data indicate the suitability of this assay for use in the identification and characterization of HRV in both laboratory and clinical settings.

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Antiviral Effect of the Combination of Enviroxime and Disoxaril on Coxsackievirus B1 Infection

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Effect s of enviroxime and disoxaril, picornavirus inhibitors with known mechanisms of action, were tested alone or in combination on the replication of coxsackievirus B1 (CVB1) in FL cells and on experimental CVB1 infection in newborn mice. The combination of enviroxime and disoxaril *in vitro* resulted in a synergistic interaction. Both compounds were administered *in vivo*, alone or in combination, daily by subcutaneous (s.c.) route since the day of virus inoculation till the 5th day post inoculation (p.i.). Our findings about the *in vivo* antiviral effects of the individual compounds correlated with those of other authors, i.e. disoxaril significantly reduced the virus-induced death (minimum 50% effective dose (ED_{50}) was 12.5 mg/kg; P=0.0037), while enviroxime was not effective even when applied at a dose as high as 100 mg/kg (P = 0.264). However, when both substances were combined, the same protective effect was achieved with concentrations of disoxaril two to four times lower that those of the drug administered alone. In this way a higher selectivity ratio was achieved. Namely, the combination of 50mg/kg enviroxime and 3.125-6.25mg/kg disoxaril was a synergistic one. Along with reduction in mortality a marked delay in the course of the disease was observed.

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Virucidal activity and cytotoxicity of a liposomal formulation of povidone-iodine

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Povidone-iodine (PVP-I) is a broad-spectrum microbicide with an excellent in vitro activity against bacteria, viruses, fungi, and protozoans. It is used in numerous topical formulations for disinfection, wound antisepsis, the treatment of burns, leg ulcers and decubital ulcers. Recently, a novel PVP-I formulation has been developed by liposomal drug encapsulation. This study was conducted to compare the virucidal activity against several viruses related to infections of the eye or the respiratory tract and the cytotoxicity of both aqueous and liposomal PVP-I formulations. Influenza A and HSV-1 were inactivated by 0.1% PVP-I formulations within 0.5 min. At PVP-I concentrations of 0.23% the exposure times necessary to inactivate adenovirus type 8 were 5 min for the aqueous and 15 min for the liposomal formulation. Human rhinovirus type 14 was inactivated by 0.45 % liposomal and aqueous PVP-I within 30 and 15 min, respectively. Half maximum cytotoxic PVP-I concentrations were 0.01-0.07% for the aqueous PVP-I and 0.03-0.27% for the liposomal PVP-I formulation. Necrosis predominates in cells treated with aqueous PVP-I, whereas apoptosis was the predominant type of cell death in cells treated with equivalent concentrations of liposomal PVP-I formulation. These results suggest that liposomal PVP-I formulations are favourable candidates for use in the prevention and treatment of infections of the eye and the upper respiratory tract.

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Characterisation of interferon alfa-2a and pegylated interferon alfa-2a in combination with ribavirin, mycophenolic acid or VX-497 as inhibitors of HCV replicon replication.

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The current treatment of HCV infection relies on alpha interferon alone or in combination with ribavirin. Until recently, the lack of *in vitro* models have prevented the assessment of novel therapies for HCV in the laboratory. To investigate the potential of IMPDH inhibition in HCV infection, especially in combination with IFN alfa-2a (IFN), we used the recently described HCV subgenomic replicon model (Lohman *et al.* Science 1999; 285: 110-113). The effects of ribavirin and two potent IMPDH inhibitors, VX-497 and mycophenolic acid (MPA), were compared alone or in combination with IFN or a 40 kDa monopegylated IFN (PEG-IFN). IFN , PEG-IFN, ribavirin, VX-497 and MPA all independently inhibited the replication of the HCV replicon in Huh7 hepatoma cells, although with differing potencies. IFN and PEG-IFN showed an IC50 of 1 IU/ml. Activity of ribavirin in this cell system (IC50 15 μM) demonstrates direct activity on replication of this HCV model system. VX-497 and MPA had similar IC50s of 0.3 μM, and reversal of inhibition by guanosine is consistent with IMPDH as the molecular target of the drugs. However, reversibility is not observed with ribavirin, consistent with it inhibiting a different molecular target in this cell line, such as the viral polymerase as suggested by others. The full data including the results from the combination experiments will be presented. These data confirm that PEG-IFN is active in this HCV model system; IMPDH inhibition has potential for the treatment of HCV infection; ribavirin may inhibit an additional target in hepatocytes; and further emphasises the benefits of the HCV replicon system for evaluation of new antiviral agents for HCV.

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The HCV-Trimera mouse: A model for evaluation of therapeutic agents against hepatitis C

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The lack of small-animal models for evaluation of therapeutic agents against hepatitis C severely hinders the assessment of potential new therapies for the management of the disease. Our goal was to create such a model, termed the hepatitis C virus (HCV) Trimera model, which could permit practical *in vivo* screening of anti-HCV agents. The HCV-Trimera model has been developed using lethally irradiated mice reconstituted with SCID-mouse bone marrow cells and transplanted with *ex vivo* HCV-infected human-liver fragments, which serve as the focus of nascent virus. The presence of (+) and (-)strand HCV RNA in mice sera and human-liver grafts was determined by RT-PCR followed by dot-blot hybridization. Viremia ((+)strand HCV RNA levels) in HCV-Trimera mice peaked around day 18 post liver transplantation, reaching an infection rate of 85%. *De novo* viral replication in liver grafts was shown by the presence of (-)strand HCV RNA. The HCV-Trimera model was used to evaluate the therapeutic effects of a small molecule (an HCV internal ribosomal entry site (IRES) inhibitor) and an anti-HCV human monoclonal antibody (HCV-AB^{XTL}68). Treatment of HCV-Trimera mice with these drugs effectively reduced both the percentage of HCV-positive animals and the viral load in their sera in a dose-dependent manner. Treatment cessation resulted in rebound of viral load, indicating HCV replication upon drug withdrawal. These results show that the HCV-Trimera model provides an *in vivo* platform technology for the evaluation of anti-HCV agents acting through different mechanisms.

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Clinical Evaluation (Phase I) of a Combination of Two Human Monoclonal Antibodies to HBV: Safety and Anti-viral Properties

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Although current treatments for chronic HBV reduce viral replication in 20-40% of patients, they frequently do not result in viral clearance. Loss of response or emergence of drug-resistant mutants have been detected with prolonged therapy. Thus, new and multiple drug approaches are needed. We have developed two fully human monoclonal antibodies (HmAb) with high affinity, directed against different epitopes of HBsAg which bind to all major HBV subtypes. Preclinical studies in a hepatitis B virus mouse model (the HBV-Trimera model) have shown that treatment of HBV infected mice with these antibodies resulted in reduction of viral loads. Similar results were obtained when treating HBV infected chimpanzees. A phase I clinical study was conducted to evaluate the safety, tolerability and efficacy of a mixture of our two HmAbs. A total of 15 patients with chronic HBV infection (HBV-DNA positive > 6 months) were enrolled. Patients were randomized to 5 groups, 3 patients in each cohort, receiving IV infusions of antibodies with doses such that the Ab:Ag molar ratio is increasing in each cohort. The doses ranged from 0.26 mg (260 IU) to 40 mg (40000 IU). All patients have completed 16 weeks of follow-up. No serious adverse events have been reported, the only one recorded was a transient mild myalgia, in 3 / 15 patients. Patients who have received doses of antibodies with Ab:Ag molar ratio of 1:2 - 1:20 have shown a rapid and significant decrease in HBsAg to undetectable levels, with a correspondent reduction of HBV-DNA levels. A rebound in HBsAg levels to pretreatment levels was observed 5 days post-infusion. **Conclusion:** Treatment of chronic HBV patients with a mixture of two anti-HBsAg HmAbs is safe and well tolerated. These mAbs are able to reduce HBV serum viral titers and HBsAg levels. These results confirm observations in mice and chimpanzees that a combination of two HmAbs may reduce HBV load. Treatment with different antibodies that are directed to more than one epitope could reduce the probability of developing escape mutations. Our mAbs may be candidates for combination therapy in HBV carriers previously treated with anti-viral monotherapy.

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Brivudin Compared to Acyclovir and Famciclovir: Effect on Zoster-Associated Pain

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Two separate investigations examined the effect of a 7-day treatment with brivudin 125 mg once daily in comparison to famciclovir and acyclovir, respectively, on zoster-associated pain (ZAP) in patients ≥50 years of age. Comparison with acyclovir: This was a double-blind post-study survey evaluating pain data obtained from 608 patients from a randomized study, lasting up to 1 month, which compared the efficacy of brivudin with acyclovir 800 mg five times daily in acute herpes zoster. Treatment started within 48 hours after first vesicular eruption. Ninety percent of the patients had pain at baseline. The incidence of chronic pain, i.e. pain (of any intensity) after study termination, was significantly lower with brivudin (32.7%) than with acyclovir (43.5%, P=0.006). The prevalence of zoster-associated pain measured in monthly intervals following study termination steadily decreased, with the difference between the treatment groups remaining constantly in favor of brivudin. Pain more than 6 months after study termination was reported by 12% of the brivudin patients and by 15.6% of the acyclovir patients. Comparison with famciclovir: The positive results of the post-study survey led to the conduct of a randomized, double-blind, multicenter study which compared the efficacy of brivudin with famciclovir 250 mg t.i.d. in 2027 herpes zoster patients. Treatment started within 72 hours after rash onset. Follow-up ranged from 3½ to 9 months. All patients had pain at baseline. With respect to the prevalence of ZAP up to month 9, equivalence of brivudin and famciclovir could be shown. In the PP population (N=1712), 21.1% of the brivudin patients and 20.2% of the famciclovir patients had ZAP (of any intensity) 3 months after start of treatment. Conclusion: Brivudin is as effective as famciclovir and significantly more effective than standard acyclovir in reducing the incidence of chronic pain. With its once daily dosing schedule, brivudin provides a considerable advantage compared to current antiviral therapies.

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Design of Papillomavirus Inhibitors Based on the Three-dimensional Structures of E6-interacting Proteins. J.D. Baleja, X. Be, J.H. Voigt, M.C. Nicklaus, J.J. Chen, and E.J. Androphy. Tufts University School of Medicine and New England Medical Center, Boston, MA USA and National Cancer Institute, Bethesda, MD, USA.

Human papillomaviruses (HPV) cause cutaneous, genital and cervical warts. A subset of the HPVs is associated with a high risk for malignant progression. The papillomavirus E6 protein represents an attractive target for therapeutic intervention because of its multiple and essential roles in viral propagation and cellular transformation. An HPV-16 E6 binding domain, called E6ap, is located on an 18-amino-acid segment of an E6-associated protein, E6AP. The corresponding peptide was synthesized and its structure was solved by nuclear magnetic resonance (NMR) spectroscopy. Three conserved leucines are observed to form a hydrophobic patch on one face of an α-helix. Substitution of any of these leucines to alanine abolished binding to E6 protein, indicating that the entire hydrophobic patch is necessary. Mutation of a glutamate to proline, but not alanine, also disrupted the interaction between E6 and E6AP protein suggesting that the E6 binding motif of the E6AP protein must be helical when bound to E6. Comparison of the E6ap structure and mutational results with those of another E6-binding protein (E6BP/ERC-55) indicates the existence of a general E6 binding motif comprising a helical structure with the most important amino acids for interaction lying on one face of the helix. We have subsequently selected, designed, and tested inhibitory compounds based on the structures and mutagenesis of the interaction domains. These investigations have provided a molecular target for the development of antiviral drugs and represent progress in creating inhibitors of E6. At the same time, the inhibitors may be used to provide mechanistic insights into the biology of the viral protein and its interaction with cellular proteins.