Oral Session I: Retrovirus Infections I

1

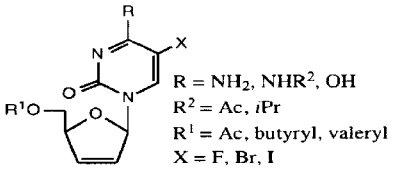
Interaction of β-L-2',3'-dideoxy-2',3'-didehydro-5-fluorocytidine 5'-triphosphate with HIV reverse transcriptase and human DNA polymerases: implication for HIV drug design. M. Kukhanova[1], X. Li[2], SH Chen[2], I. King[2], T. Doyle[2], W. Prusoff[1], and Y.C Cheng[1]. [1]Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510; [2]Vion Pharmaceutical Inc, New Haven, CT 06511

This study used DNA primer extension and sequencing gel analysis to evaluate the relative molecular activity of the 5'-triphosphate of a novel β-L-nucleoside with an unsaturated ribose residue, β-L-2',3'-dideoxy-2',3'-didehydro-5-fluorocytidine (β-L-Fd4CTP), with β-L-2',3'-dideoxy-5-fluorocytidine (β-L-FddCTP) and ddCTP, on DNA strand elongation by HIV reverse transcriptase and human DNA polymerases pol α, pol β, pol γ, and pol ε. The concentrations of β-L-Fd4CTP that inhibited the yield of products by 50% were 0.20 ± 0.05 μM, 1.8 ± 0.5 μM, 4 ± 1 μM for HIV RT, pol γ, and pol β, respectively. β-L-Fd4CTP at a concentration as high as 40 μM had no inhibitory effect on poly ε, but could inhibit pol α by 10-20% at 30 μM. The Km and relative V max values of β-L-Fd4CTP, β-L-FddCTP and ddCTP for incorporation into the standing point of DNA by HIV reverse transcriptase and human DNA polymerases in a system containing M13mp19 phage DNA annealed with 5'-^{32}P-oligonucleotide primer were evaluated. The efficiency of incorporation (Vmax/Km) of β-L-Fd4CTP by HIV reverse transcriptase was about four times and one order of magnitude higher than that of ddCTP and β-L-F-ddCTP, respectively. In contrast, Vmax/Km ratio of β-L-Fd4CTP for pol γ was 6 times lower than that of ddCTP, but four times higher than that of β-L-FddCTP. Pol α could use β-L-Fd4CTP as a substrate, but only at a high concentration (> 40 μM). Incorporation of β-L-Fd4CTP by pol ε could not be detected. A hypothesis has been discussed about the preferable recognition of the 2',3'-dideoxy-2',3'-didehydro - structure of β-L-Fd4CTP to that of the 2',3'-dideoxy-structure of β-L-FddCTP. Supported by NIH grant AI-38204.

2

Synthesis and *In Vitro* Anti-HIV-1 Activity of D-D4FC Analogs. J. Shi,[1,2] D.C. Liotta,[3] and R.F. Schinazi.*[1,2] Dept. of Pediatrics[1] and Chemistry,[3] Emory University, and VA Med. Ctr.,[2] Atlanta, GA 30033.

The discovery of a novel cytosine nucleoside, β-D-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (D-D4FC) as a potent anti-HIV agent lead us to synthesize a series of analogs and prodrugs that could increase the stability of the glycosidic bond. The N^4-alkyl, acyl, and 5'-*O*-acyl derivatives, and 5-halogen, uracil, and carbocyclic analogs were synthesized and evaluated *in vitro* for anti-HIV-1 activity and cytotoxicity. The 5'-*O*- and N^4-acyl derivatives demonstrated activity comparable to D-D4FC, whereas the N^4-*i*Pr derivative was devoid of activity even at 100 μM. The 5-halogen cytosine and 5-fluorouracil analogs were not active, except for β-L-D4FU which showed potent *in vitro* anti-HIV-1 activity and cytotoxicity. The carbocyclic analogs demonstrated weak anti-HIV-1 activity and no cytotoxicity. *(Supported by NIH grant AI-41980, AI-28731, and VA)*



3

L-PURINE DIDEOXYNUCLEOTIDE PRODRUGS AS INHIBITORS OF HIV REPLICATION. L-L. Imbach,[1] G. Gosselin,[1] C. Mathé,[1] C. Pierra,[1] V. Boudou[1], A. Faraj[2], E. Cretton-Scott,[2] L. Placidi,[2] R.F. Schinazi,[3] and J.-P. Sommadossi[2].
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The unnatural β-L-2',3'-dideoxyadenosine (β-L-ddA) and other L-purine nucleoside derivatives lack substantial antiviral activity against human immunodeficiency virus (HIV) replication in PHA-activated human peripheral blood mononuclear (PBM) cells with EC_{50} values > 100 μM. Of importance, β-L-ddA was not phosphorylated to its 5'-triphosphate in human PBM cells and was rapidly catabolized through the cleavage of the glycosidic bond despite being a poor substrate towards adenosine deaminase. β-L-ddA was also glucuronidated in primary human cultured hepatocytes. The demonstration that the 5'-triphosphate derivative of β-LddA (β-L-ddATP) potently and selectively inhibited HIV-RT activity with a K_i of 2.0 μM while it had no effect on human DNA polymerases α, β, and γ up to a concentration of 100 μM, suggested that stabilized prodrugs of L-ddAMP could exert potent and selective anti-HIV activity. Among several prodrugs, β-L-ddAMP-bis-(tbutylSATE) potently inhibited HIV replication with an EC_{50} of 0.002 μM in HIV-infected human PBM cells. In addition, a combination of β-L-ddAMP-bis-(tbutylSATE) and other nucleoside analogs demonstrated synergistic activity against HIV replication *in vitro*. Intracellular metabolism of [^{3}H] β-L-ddAMP-bis-(tbutylSATE) demonstrated that the predominant metabolite β-L-ddATP achieved a concentration of 20- to 40-fold higher than that of 3TC-TP under similar conditions in human PHA-stimulated PBM cells. The intracellular half life of β-L-ddATP was approximately 10 hr with levels still above the K_i for as long 48 hr after the removal of the drug. In conclusion, these data demonstrate that β-L-ddAMP-bis-(tbutylSATE) is a potent and selective anti-HIV agent and its very significant *in vitro* synergy with other nucleoside analogs supports its further development.

4

PETT-4, New Potent Allosteric Inhibitors of HIV-1 Reverse Transcriptase

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Further development of trovirdine (PETT-1) resulted in PETT-4, a group of potent allosteric inhibitors of HIV-1 reverse transcriptase (RT). Among these are compounds possessing anti-HIV-1 activity in cell culture better than that of nevirapine, delavirdine, HBY 097 and DMP 266 and at concentrations below 1nM. A high antiviral potency is also observed in the presence of human serum and the RT mutations L100I and K103N. PETT-4 compounds show a slower rate of resistance development in vitro than nevirapine, delavirdine, HBY 097 and DMP 266. Plasma levels of PETT-4 above ED_{50} for the first mutant in cell culture can be observed for more than 8h after oral dosing to rats. Penetration to the brain of rats occurs at levels equal to plasma levels. PETT-4 compounds are highly effective against SHIV (SIV with HIV-1 RT) in monkeys. A PETT-4 development program for an IND is ongoing with one selected compound.

5

Crystal Structures of the PETT Compounds MSC-204 and MSH-372 in Complex with Human Immunodeficiency Virus Reverse Transcriptase

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The PETT compounds MSC-204 and MSH-372 were co-crystallized with human immunodeficiency virus reverse transcriptase (HIV-1 RT). Crystal structures were determined to 2.7 A resolution with an R-value of 25%. The RNase H mutant RTE489Q was used as the target molecule and the structure determined by molecular replacement. The inhibitors bound to the allosteric non-nucleoside RT inhibitor binding pocket. MSC-204 and MSH-372 both fill out the pocket efficiently making close contacts to the aromatic residues through π-π interactions. The PETT compounds contain stabilizing internal hydrogen bond interactions. The very good fits of MSC-204 and MSH-372 to the allosteric binding pocket are consistent with the potent inhibitory activities of these compounds.

6

Involvement of an Active Efflux Pump in the Cellular Resistance of Anti-Retroviral Nucleoside Analogs. A Fridland, SG PaiBir, RV Srinivas, MC Connelly, BL Robbins, FH Pinkerton, JD Schuetz. St. Jude Children's Research Hospital, Memphis, TN, USA.

We have previously reported a human T cell line, CEM_{ss}-r1, that is resistant to the acyclic nucleoside phosphonate analog PMEA (Mol Pharmacol 47:391). The antiviral action of AZT and 3TC was also reduced in the CEM_{ss}-r1 cell. This multidrug resistance phenotype is associated with an cellular efflux of the phosphonate or AZT metabolite. The objective of this study was to identify and characterize the efflux pump. Initial biochemical studies indicated that the efflux of the compounds was ATP-dependent suggesting the involvement of an efflux pump belonging to the ABC superfamily of transport proteins. However, modulators of P-glycoprotein (P-gp), such as verapamil and colchicine did not have any effect on the efflux of the nucleotide analogs, showing that P-gp does not contribute to the resistant phenotype of CEM-r1 cells. However, probenecid and dipyridamole, known inhibitors of multispecific organic anion transport, inhibited the efflux and increased the accumulation of the nucleotide analogs in CEMr-1 cells, but not wild type. In conclusion, these results demonstrate the presence of an anion carrier in human T cells that modulate the intracellular level of nucleotide analogs and may be important to the antiviral efficacy of these agents.

7

Comparison of the Antiviral Activity of Several Acyclic Nucleoside Phosphonate Derivatives in Feline Immunodeficiency Virus-Infected Cats

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The acyclic nucleoside phosphonates (S)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA), (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA), (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine (PMPDAP), and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were compared for their efficacy in a placebo-controlled double-blind trial in naturally feline immunodeficiency virus (FIV)-infected cats. This natural animal model is considered as highly relevant for the pathogenesis and chemotherapy of HIV infection in humans. FPMPA, PMPA, PMPDAP, and PMEA were used in this study because they show marked anti-FIV activity in cell culture. Seventy-five naturally FIV-infected cats were included in the study. All the acyclic nucleoside phosphonates proved effective in ameliorating the clinical symptoms and the immune status of the FIV-infected cats as compared to the placebo-treated animals, as measured by several clinical parameters including the incidence and severity of stomatitis, Karnofsky's score, immunologic parameters such as relative and absolute $CD4^+$ and $CD8^+$ cell counts, neopterin and other pterin values in plasma and urine, and virologic parameters including proviral DNA levels in peripheral blood mononuclear cells. PMEA was superior to the other compounds in improving the clinical symptoms but showed more hematological side effects at a drug dose that was 2.5-fold lower than the dose of FPMPA, PMPA and PMPDAP.

8

Can the Bottleneck in AZT Activation be Efficiently Circumvented by Transfection of HIV-Susceptible Cells with Murine Thymidylate Kinase or Herpetic Thymidine Kinase ?

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It has been shown that the phosphorylation of AZT to its eventual active metabolite AZT 5'-triphosphate (AZT-TP) in human H9 cells occurs at a low efficiency and is due to the inefficient conversion of AZT 5'-monophosphate (AZT-MP) to AZT-DP, by human thymidylate (dTMP) kinase (Furman et al., Proc. Natl. Acad. Sci. 83: 8333-8337, 1986). We have extended the original observation to a variety of other human cell lines including ATH8, Molt4, CEM, HeLa, hepatoma and osteosarcoma cells, as attested by the intracellular accumulation of AZT-MP (AZT-DP+AZT-TP to AZT-MP ratios ranging from 0.13 to 0.88). Interestingly, murine cells (i.e. leukemia L1210, breast carcinoma FM3A and embryo fibroblast L929) invariably showed AZT-DP+AZT-TP to AZT-MP ratios that were markedly higher than 1 (between 5 and 12). Thus, in contrast with human dTMP kinase, murine dTMP kinase does not represent a bottleneck in the eventual intracellular conversion of AZT to AZT-TP. Since herpes simplex virus type-1 and varicella-zoster virus thymidine kinase have been previously shown to possess associated dTMP kinase activity as well, we have also measured the phosphorylation pattern of AZT in HSV-1 and VZV TK gene-transfected cells and found AZT-DP+AZT-TP to AZT-MP ratios that amounted up to 17 and 32. Thus, herpes virus-encoded dTMP kinase-associated TK is able to convert AZT-MP to AZT-DP at a markedly higher rate than human dTMP kinase. Therefore, both the murine dTMP kinase gene and HSV or VZV TK genes may be considered as candidate genes for combined dTMP kinase (or TK) gene/AZT chemotherapy of HIV-infected cells.

9

Genotypic Characterization of HIV-1 Isolated from AIDS Patients After Prolonged Therapy with Adefovir Dipivoxil (Preveon™) Added to Existing Regimens. J.M. Cherrington, P.D. Lamy, N.A. Margot and A.S. Mulato. Gilead Sciences, Foster City, CA. 94404.

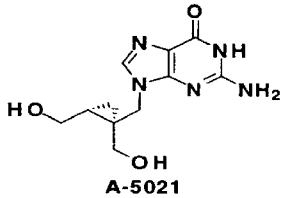
Adefovir dipivoxil (bis-POM PMEA; Preveon™) is currently in clinical trials for the treatment of HIV infection. In an earlier Phase II study of extended Preveon therapy in which concomitant antiretrovirals were permitted, HIV RT genotypic analyses revealed that 8 of 29 patients developed genotypic changes in RT in association with Preveon treatment. Importantly, all 8 of these patients experienced sustained viral load suppression during the dosing period of 6-12 months. In a current Phase III study patients were randomized to add Preveon or placebo to their existing antiretroviral regimens for 24 weeks; drug was then administered open label during weeks 24-48. The majority of patients had received $\geq$ 3 nucleosides previously. Genotypic analyses of baseline and week 24 HIV RT genes from 64 of 442 patients enrolled demonstrated that baseline samples commonly carried numerous AZT-resistance mutations and the 3TC-associated M184V mutation. RT changes from baseline that were not attributed to concomitant medications were identified at week 24 in 9 of these 64 patients. Two patients developed a T69S mutation, 1 a T69N and the remaining 6 developed AZT-resistance mutations. This study is still blinded so it is not yet possible to determine whether Preveon therapy selected these changes. RT sequence data from 39 of the 64 patients during the open label phase of the study identified 6 more patients who developed changes from baseline at $\geq$ 32 weeks: 5 with AZT-resistance mutations and 1 with a T69S mutation and a double serine insertion between RT amino acids 69 and 70. ddI and d4T therapy also have been shown to select for AZT-resistance mutations previously. The resistance patterns described here are similar to those observed in the Phase II study and confirm that mutations associated with Preveon resistance do not arise readily during prolonged therapy. The effects of baseline and week 24/48 RT genotypes on virological response to Preveon will be presented.

Oral Session II: Herpesvirus Infections I

10

A Novel Potent Anti-VZV Pyrimidine Nucleoside **AV-100**: Synthesis, Antiherpetic Activity and Pharmacokinetics. T. Tsuji, T. Onishi, C. Mukai, R. Nakagawa, T. Sekiyama, M. Aoki, K. Suzuki, H. Nakazawa, N. Ono, Y. Ohmura, S. Iwayama, and M. Okunishi Ajinomoto Co. Inc. Kawasaki JAPAN

A series of pyrimidine derivatives of **A-5021**, (1'*S*, 2'*R*)-9-[[1',2'-bis(hydroxymethyl)cycloprop-1'-yl]methyl]guanine, were synthesized and evaluated for their anti-herpetic activities. Among the compounds synthesized, 5-(*E*)-halovinyluracil derivatives showed superior anti-VZV activity over ACV while less potent than ACV against HSV-1. IC_{50} values for VZV Kawaguchi strain are 0.031 for Br, 0.07 for Cl, 0.054 for I derivatives, 0.34 for **A-5021**, 3.4μg/ml for ACV, respectively. The most potent compound, **AV-100**, (1'*S*, 2'*R*)-5-[(*E*)-2-bromoethenyl]-1-[[1',2'-bis(hydroxymethyl) cycloprop-1'-yl]methyl]-2,4-(1*H*, 3*H*)-pyrimidinedione, is 40- to 60-fold more potent than ACV against clinical isolates of VZV. **AV-100** showed good oral bioavailability in rats (68.5%). Unlike BVaraU, no release of BVU, a potent dihydropyrimidine dehydrogenase inhibitor, was observed in the plasma. No toxic symptoms were observed in mice up to 240mg/kg/day when administered intravenously for 6 days. Because of its potent anti-VZV activity, superior oral bioavailability, and safety profile, **AV-100** is expected as a clinical candidate for the treatment of VZV patients.



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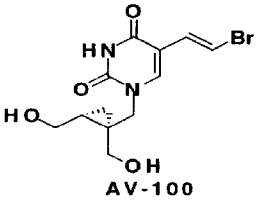

AV-100

11

Aminothiazolyl-Phenyl-Based Inhibitors of HSV Helicase-Primase: A Novel Class of Orally Active Antiherpetic Agents

M. Liuzzi, J.J. Crute, C.A. Grygon, K.D. Hargrave, B. Simoneau, A.-M. Faucher, G. Bolger, J. Duan, P. Kibler and M.G. Cordingley. Bio-Méga Research Division, Boehringer Ingelheim (Canada) Ltd., Laval, Quebec, Canada H7S 2G5; Department of Immunological and Inflammatory Diseases, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT 06877-0368.

The herpes simplex virus (HSV) type 1 helicase-primase is a heterotrimeric enzyme composed of the UL5, UL8 and UL52 gene products and possesses DNA helicase, DNA-dependent ATPase and primase activity. All three subunits of the complex are required for viral DNA replication and are essential for virus growth. UL5 is the helicase subunit as it contains six motifs conserved in superfamily I of known helicases whereas the primase active center has been localized within the UL52 subunit. UL8 has been implicated in modulating these activities, facilitating nuclear uptake of the complex and interacting with other viral DNA replication proteins. Using a high throughput helicase assay we have identified aminothiazolyl-phenyl-containing compounds that are specific inhibitors of the HSV helicase-primase. Optimization of lead inhibitors yielded the compound BILS 45 BS. This compound inhibited all three activities of the enzyme at concentrations less than 1 μM and exhibited potent antiviral activity against a series of wild-type and acyclovir-resistant HSV-1 and HSV-2 strains in cell culture. Most importantly, BILS 45 BS was orally active against wild-type and ACV-resistant HSV infections in experimental animal models of HSV disease. Marker rescue of HSV-1 resistant to aminothiazolyl-phenyl containing inhibitors indicated that resistance mutations mapped to the UL5 gene. DNA sequence analysis identified single amino acid substitutions clustered near the N-terminus of UL5 adjacent to the conserved helicase motif IV of the helicase superfamily I. These results demonstrate that the mechanism of antiviral action is through inhibition of the HSV helicase-primase. Taken together, the data indicate that aminothiazolyl-phenyl-based inhibitors of the HSV helicase-primase are effective in the treatment of HSV disease and constitute therefore potential candidates for the development of clinically useful antiherpetic agents.

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T157602, A 2-Amino Thiazole Inhibits HSV Replication by Interacting with the UL5 Component of the UL5/8/52 Helicase Primase Complex

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Utilizing a high throughput biochemical helicase assay as a screen T157602, a 2-amino thiazole compound was identified as a specific and potent inhibitor of HSV replication. T157602 reversibly inhibited the helicase activity of the HSV UL5/8/52 helicase:primase complex with an IC50 of 5uM. The inhibition was found to be very specific with respect to other helicases. The primase activity of the UL5/8/52 complex was also inhibited by T157602 with an IC50 of 20uM. T157602 inhibited HSV growth in a one step viral growth assay with an IC99 of 6uM and plaque formation was completely prevented at concentrations of 25-50uM T157602. Vero, HFF and Jurkat cells could be propagated in the presence of T157602 at concentration exceeding 100uM with no obvious cytotoxic effects, indicating that the window between antiviral activity and cellular toxicity is at least 33-fold. Four independently derived HSV-2 T157602 resistant mutants and three T157602 resistant HSV-1 viruses were generated and plaque purified. All of the T157602 resistant viruses carried single base pair mutations in the UL5 gene resulting in single amino acid changes in the UL5 protein. Marker rescue experiments demonstrated that the UL5 gene from T157602 resistant viruses could confer resistance to T157602 sensitive wild type viruses. Finally, purified UL5/8/52 helicase:primase complex carrying the specific point mutation in the UL5 protein showed complete resistance to T157602 in a helicase assay. T157602 and its analogs represent a novel class of specific and reversible anti-HSV agents that elicit their inhibitory effects on HSV replication by interacting with the UL5 component of the viral helicase:primase complex.

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Complementation of a thymidine kinase deficient mutant of herpes simplex virus type 1 by the parent wild type virus in a zosteriform model of herpes virus infection in mice.

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Acyclovir resistant, thymidine kinase deficient variants of herpes simplex virus (HSV) are the most frequent drug resistant variants encountered in the clinic and due to their lack of or limited ability to replicate in neuronal cells, generally demonstrate diminished neurovirulence. Clinical virus isolates may not be clonal and as such, thymidine kinase deficient clinical isolates may contain low levels of wild type virus. The aim of this study was to investigate the importance of complementation of thymidine kinase deficient HSV infection, by wild type virus, in a zosteriform model of HSV infection in Balb/c mice. Following a challenge of 1×10^5 pfu of wild type HSV type 1, strain SC16, on the necks of mice, virus replicated at the site of inoculation, and following neuronal spread, virus was subsequently recovered from the cervical ganglia and at a secondary peripheral site, the ipsilateral ear pinna. Virus was only recovered from the primary peripheral site following infection with HSV type 1, strain DM21, an acyclovir resistant, thymidine kinase deletion mutant of strain SC16. However, mice inoculated with a mixture of 1×10^5 pfu of strain SC16 and 4.8×10^6 pfu of strain DM21 demonstrated strain DM21 replication in the cervical ganglia and ipsilateral ear pinna at levels comparable with those for strain SC16. Even when the strain SC16 challenge in the mixture was reduced to 1×10^3 pfu, low levels of strain DM21 were still recovered from the ganglia and ear pinna in almost equal quantities to strain SC16. These data confirm that wild type virus will complement thymidine kinase deficient HSV infection in an experimental disease model in mice, even at low levels of wild type virus challenge.

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HSV Latency Detected In Neurons by Means of the β-gal Reporter Gene in Ganglia from Mice Treated with Famciclovir or Valaciclovir.

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Previously we reported that early chemotherapy using famciclovir (FCV) has a marked effect on the reactivation of latent HSV from ganglia using a murine ear pinna infection model. Thus, ganglia from mice treated within a few days of inoculation failed to yield infectious virus after cocultivation (Thackray & Field, 1996, JID, **173**: 291-299). It was shown, however, that latent foci were detectable using the more sensitive techniques of enzymatic disaggregation or in situ hybridisation. In the present experiments a recombinant virus (SC16 LβA) which contains the β-galactosidase reporter gene under the LAT promoter (Lachmann & Efstathiou, 1997, J. Virol, **71(4)**: 3197-3207) was used to study the effects of early treatment on the establishment of latency. 350 mice were infected with LβA or wild-type virus. During the acute infection the recombinant exhibited similar pathogenicity to the wild-type virus. Mice were treated from 1 day before or 1 day after virus inoculation using 1mg/ml of FCV or valaciclovir (VACV) in the drinking water. Mice were killed on days 28, 38, 40, 43, 73, 77 and 81 and their dorsal root ganglia, trigeminal ganglia and brain stems were studied for the presence of latent infection using four different methods. Sections stained with X-gal clearly showed the presence of the reporter gene in the cytoplasm of neurons from mice inoculated with the recombinant virus. The number of latently infected neurons as judged by the 4 different methods was markedly reduced in FCV-treated mice. However, it was notable that, using the sensitive X-gal method, a residual number of neurons stained positive even when therapy was commenced one day before virus inoculation. It is speculated that this basal level of infected cells may represent virus entering axons direct from the inoculum.

15

Assessment of the Relationship Between the Skin Target Site Free Drug Concentration (C*) and the *In vivo* Efficacy for Bromovinyldeoxyuridine and Acyclovir Formulations in the Treatment of Cutaneous HSV-1 Infections in Hairless Mice.

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Previously we have investigated the relationship between the skin target site free drug concentration (C*) determined from the *in vitro* flux experiments and the *in vivo* antiviral efficacy for variety of the formulations containing 5% Acyclovir (ACV). In the current study, we examined the relationship between the C* predictions and the *in vivo* efficacies for some topical formulations containing different concentrations (0.05%-10%) of either ACV or Bromovinyldeoxyuridine (BVDU) in 95% DMSO as a vehicle with or without 5% Azone as skin permeation enhancer. Hairless mice infected cutaneously with HSV-1 were used to quantitatively estimate the *in vivo* topical antiviral efficacy. A finite dose of the test antiviral formulation was applied twice a day for four days, starting the day after virus inoculation. On the fifth day, the lesions were scored and the efficacy values were calculated as described earlier (Gonsho *et al., Int. J. Pharm.*, 65, 183-194 (1994)). For each formulation *in vitro* flux experiments were performed in an *in vivo-in vitro* experimental design that closely approximated the *in vivo* study protocol. As was previously shown, with all ACV formulations, a good correlation was found between the C* predictions and the *in vivo* topical efficacy (i.e., topical efficacy was found to be a single-valued function of C*). With the BVDU formulations, on the other hand, this was found not to be the case. BVDU formulations with 5% Azone were generally much more effective than those without Azone for the same C*. This finding is believed to be the first of its kind showing that skin "permeation enhancers" may enhance efficacy by more than simply increasing skin permeation rates. Supported by a Grant-in-Aid from Thera Tech, Inc., by NIH Grant A1 20161 and by the Egyptian Government.

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Acyclovir-resistant Herpes Simplex Virus: Preliminary Results from a National Surveillance System

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Despite increasing reports of acyclovir (ACV) failures for the treatment of herpes simplex virus (HSV), the prevalence and risk factors for ACV-resistant HSV remain poorly defined. **Methods**: We established a pilot surveillance system to estimate the prevalence of ACV-resistant HSV and examine risk factors. HIV+ and STD clinic patients (pts.) were enrolled from 22 study sites in 12 US cities. Swab specimens of lesions were cultured for HSV and isolates were tested for ACV-sensitivity by plaque reduction assay. History of HSV disease and treatment, sexual activity, and HIV status were determined by interview. **Results:** Of 1736 pts., 888 (51.2%) tested positive for HSV-2 and 99 (5.7) for HSV-1; 979 (99.2%) were sensitive to ACV (cut-off point 2 *ug*/ml; median 0.57 *ug*/ml; range 0.01-1.99). Eight ACV-resistant isolates, (median IC50 15.6 *ug*/ml; range 4.5-42.4), 7 (5.6%) were from 126 HIV+ pts., and 1 (0.1%) was from 861 STD clinic pts. Factors associated with a resistant isolate were: ever having taken ACV ($p<0.001$); currently taking ACV ($p<0.001$); use of topical ACV for the current outbreak ($p<0.02$); duration of the current HSV lesion ($p<0.02$), and HIV+ status ($p<0.001$). Pts. taking ACV for a prolonged course (avg. 134 days) were less likely ($p<0.001$) to have a resistant isolate than those taking a shorter course of therapy (avg. 20 days). Anecdotally, the one STD pt. with a resistant isolate took "one pill (ACV) a day for 60 days." Age, race, ethnicity, sex, or number of sex partners in the last year were not statistically associated with ACV-resistance. **Conclusions:** These ACV-resistance estimates (5.6% among HIV+ and 0.1% among STD clinic pts.), though preliminary, are consistent with other reports. The analysis of risk factor data indicate that HIV+ status and use of ACV are important predictors for the development of ACV resistant HSV. Further analysis of these data are necessary to further explore the relationship of dose and suboptimal dosing with ACV. Continued surveillance is essential to obtain a more precise estimate of ACV resistance and ascertain if the prevalence is changing over time. Additionally, more in-depth studies are needed to characterize the clinical and public health significance of ACV resistance.

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Acyclovir-resistant Herpes Simplex Virus: Characterization of virus isolates from a nationwide surveillance system. N.T. Wetherall[1], C.A. Hodges-Savola[1], M. Reyes[2], J.M. Graber[2], W. Lawrence[3], S. Nehls[3], E. Harden[4], E.R. Kern[4], and the Task Force on Herpes Simplex Virus Resistance. ViroMed Laboratories, Inc.[1], Minneapolis, MN, Centers for Disease Control[2], Atlanta, GA, Glaxo Wellcome, Inc.[3], RTP, NC, and University of Alabama at Birmingham[4], Birmingham., AL.

Acyclovir (ACV) has been used to treat Herpes Simplex Virus (HSV) infections for almost two decades. During this time, many of the reported ACV resistant (ACVr) isolates were obtained from immunocompromised hosts and found to be thymidine kinase deficient (TK-). A collaborative multi-institutional study has been established to monitor HSV resistance. Specimens from genital lesions were sent to a central laboratory where they were cultured for HSV in primary rabbit kidney cells. Sensitivity to ACV was evaluated by plaque reduction assay (PRA) in Vero cells. Isolates exhibiting *in vitro* resistance ($EC_{50} \geq 2.0$ µg/mL ACV) were forwarded to other laboratories where they were evaluated for ACVr by PRA in Vero cells and human foreskin fibroblasts, mouse pathogenicity and DNA sequencing. Of 2334 specimens cultured during the first eleven months of the program, 1338 (57.3%) were positive for HSV; of these, 1194 (89.2%) were ACV-sensitive HSV-2 isolates, 134 (10.0%) were ACV-sensitive HSV-1 isolates and 10 (0.7%) were ACVr HSV-2 isolates. Nine of these resistant isolates were confirmed ACVr by other laboratories. When tested against other antiviral agents, 9/9 isolates exhibited cross resistance to penciclovir and 6/9 isolates were resistant to ganciclovir. In contrast, 9/9 isolates were sensitive to cidofovir and foscarnet. Neurovirulence was markedly reduced in mice inoculated with 8/9 ACVr isolates. To date, 7/9 ACVr isolates have been sequenced and 4 have been found to have a premature stop codon within the TK coding sequence. It is noteworthy that mutations within conserved regions of the TK which have been identified as ACVr are present in only 1 of these characterized isolates. These and other findings suggest that this program possesses the essential design and specimen numbers to identify unique HSV ACVr isolates, and possibly other antiviral drug resistant isolates, from both immunocompetent and immunocompromised individuals.

Oral Session III: Hepadnavirus and Papillomavirus Infections

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The Effects of the ß-*D* and ß-*L* Enantiomers of 2',3'-dideoxy-2',3'-didehydro-5-fluorocytosine Triphosphate (D4FC-TP) on the Synthesis of Duck Hepatitis B Virus (DHBV) primer DNA In Vitro. K. A. Staschke[1], R. F. Schinazi[2], X. Shi[2], D. C. Liotta[2], and J. M. Colacino[1]. [1]Lilly Research Laboratories, Indianapolis, IN 46285; [2]Emory University School of Medicine/VA Medical Center, Decatur, GA 30033.

Similar to retroviruses, the synthesis of hepadnaviral minus-strand DNA by the viral reverse transcriptase proceeds in a discontinuous fashion. In the case of DHBV, a short four nucleotide primer (5'-GTAA-3') is synthesized using the bulge of the stem-loop packaging signal (ε) as the template. Following an intramolecular template-switch, minus-strand DNA synthesis resumes from a direct repeat (DR1) on pregenomic RNA. This process can be reconstituted *in vitro*, using a cell-free translation system as the source of active reverse transcriptase. With this system, we examined the effects of *D*- and *L*-enantiomers of D4FC-TP on the formation of DHBV primer DNA. Utilizing a site-directed mutant of the bulge-loop of ε, the ability of various 2',3'-dideoxycytidine triphosphate analogs to inhibit the formation of DHBV primer DNA having the sequence 5'-GTAC-3' was examined. The triphosphate of the "unnatural" *L*-enantiomer of D4FC displayed a concentration-dependent inhibition of ^{32}P-dCTP incorporation into primer DNA with an IC_{50} of 8.5 µM. In contrast, the triphosphate of the "natural" *D*-enantiomer had no effect with an IC_{50} of > 100 µM. For comparison, two other deoxycytidine analogs, 3TC-TP and *D*-ddC-TP, displayed IC_{50}s of 51.8 µM and 39.4 µM, respectively. The activity of *L*-D4FC was examined against a mutant of DHBV polymerase which contains an amino acid substitution of lysine to arginine at position 395 (K395R). This substitution is analogous to the K65R mutation which is found in the "fingers" sub-domain of HIV-1 reverse transcriptase that is resistant to various nucleoside analogs including 3TC, *D*-ddC, PMEA, and DXG. The mutant DHBV DNA polymerase was approximately 4.5-fold less sensitive to *L*-D4FC (IC_{50} = 38.1 µM). Both the *D*- and the *L*-enantiomers of D4FC are potent inhibitors of HIV and HBV in cell culture. However, *D*-D4FC was found to be inactive against DHBV in both avian and human cells. Taken together, these results suggest that DHBV reverse transcriptase, which displays a high degree of homology to HIV and HBV reverse transcriptase in the RT domain, is naturally resistant to *D*-D4FC. (RFS and DCL are supported by NIH Grants AI-41980 and AI-28731 and the VA).

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Effect of Oral (-)-β-2′,3′-Dideoxy-5-Fluoro-3′-Thiacytidine [(-)-FTC] In Carriers of Infected Woodchuck Hepatitis Virus (WHV). Schinazi, R.F.[1*] Liotta, D.C.,[1] Hurwitz, S.J.,[1] Painter, G.,[2] Furman, P.,[2] Barry, D.,[2] Korba, B.E.,[3] and Tennant, B.C.[4] Emory Univ. Sch. of Med./VA Medical Center, Decatur, GA 30033;[1] Triangle Pharmaceutical, Inc., Durham, NC 27707;[2] Georgetown Univ. Mol. Virology and Immunol., Rockville, MD 20852;[3] and Cornell Univ. College of Veterinary Med., Ithaca, NY 14853.[4]

(-)-FTC is a nucleoside analog with highly selective anti-HBV and HIV activity *in vitro*. Recently, (-)-FTC was shown to have potent antiviral activity in HIV infected individuals. Previous studies have demonstrated that (-)-FTC was effective at 20 and 30 mg/kg (BID) when given intraperitoneally to WHV infected woodchucks (*AAC* **41**:2076-2082, 1997). We wished to determine the optimal *oral* dose of (-)-FTC necessary to inhibit viral production in serum and liver of chronically WHV infected woodchucks prior to studies in HBV infected individuals. Five groups of 4 woodchucks each were treated orally with (-)-FTC at 0.3, 1, 3, 10, and 30 mg/kg per day for 4 weeks. One group of four served as control and was not treated. (-)-FTC was effective at reducing virus production in serum at doses of 3, 10, and 30 mg/kg, but not at 0.3 and 1 mg/kg. Rapid and almost complete virus depletion was noted by day 7 at the highest dose evaluated. Lag times for virus depletion were inversely related to dose; the estimated terminal $t_{1/2}$ of virus in serum in these animals was 0.73 days. When treatment was discontinued, virus rebounded to pretreatment levels. No untoward effects were noted in any of the drug-treated groups. The results indicate that oral (-)-FTC reduces WHV in a dose dependent manner in woodchucks. (Supported by NIH grants AI-41980 and AI-28731, NO1-AI-35164, and the Dept. of Veterans Affairs).

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Decreased Replication Capacity of Duck Hepatitis B Virus Polymerase Gene Mutants Resistant to Lamivudine

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Longterm administration of Lamivudine for the therapy of chronic HBV infection is associated with the emergence of resistant mutants in 15-30% of the patients after 1 year. The major mutations reside in the YMDD motif of the viral reverse transcriptase. To study the biology of these HBV mutants, their replication capacity and pathobiology have been analyzed in the duck HBV model. Three mutants, known to confer resistance to 3TC in HBV infected patients, were engineered by site-directed mutagenesis in the DHBV genome. Mutations of the viral polymerase and their consequences on the overlapping S gene are shown below : Wild type (WT): YMDD (Pol ORF), LIWM (S ORF); Mutant 1 : YIDD (Pol ORF), LIID (S ORF); Mutant 2: YIDD (Pol ORF), LISM (S ORF); Mutant 3: YVDD (Pol ORF), LMWM (S ORF). Expression of the different viral polymerase polypeptides in a rabbit reticulocyte system and further analysis of reverse transcriptase activity *in vitro* showed a processivity defect of all 3 mutants compared to the WT polymerase. These mutants were further cloned in a vector allowing the expression of the viral pregenome under the control of the CMV promoter. Analysis of viral replication capacity was performed after transient transfection of LMH cells. Southern blot analysis of intracellular core DNA showed a more than 10 fold decrease of viral DNA replicative intermediates while expression of core proteins was not affected. The same constructs were studied *in vivo*, in comparison with a WT virus expression vector, after intrahepatic transfection of 16 ducklings in the absence of Lamivudine. The results showed that all mutants reverted to WT polymerase sequence. Our data imply that mutations in the YMDD motif of the DHBV polymerase, confering resistance to Lamivudine, affect viral replication *in vitro* and *in vivo*, via a processivity defect of the viral reverse transcriptase. This defect may explain the reversion to wild type observed *in vivo* in the absence of antiviral pressure.

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Antiviral Activity of a Novel L-Nucleoside Analog, β-L-FD4C, in the Duck HBV Infection Model.

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2',3'-dideoxy-2',3'-didehydro-β-L-5-fluorocytidine, L-FD4C, a novel L-nucleoside analog of cytidine, was recently shown to be a strong inhibitor of hepatitis B virus (HBV) replication in hepatoma cells (F2215). We have therefore evaluated its antiviral activity in the duck HBV model. The *in vivo* efficacy and toxicity of L-FD4C were assessed in experimentally infected ducklings, in comparison with Lamivudine, L-FddC and ddC. Birds received the drugs by i.p. administration for 5 days, at a dose of 0.2 mg/kg/d (5 animals/drug), 2 mg/kg/d (5 animals/drug), 5 mg/kg/d (6 animals/drug) and 25 mg/kg/d (8 animals/drug). 19 more animals served as controls. At each dose, L-FD4C was the more effective compound. The inhibition of the peak of viremia was maximum at a dose of 25 mg/kg and reached 52% for ddC, 82% for Lamivudine, 87% for L-FddC and 97% for L-FD4C. However, reappearance of viremia was observed after drug withdrawal. At the end of therapy, analysis of liver histology showed more hepatocyte necrosis in the ddC group; 2 weeks after cessation of therapy, microvesicular steatosis was found predominantly in the ddC and Lamivudine groups, while no difference was observed in L-FD4C treated animals compared to the controls. Using a cell free system for the expression of an enzymatically active DHBV reverse transcriptase, L-FD4C-TP was shown to exhibit a concentration dependent inhibition of dCTP incorporation in viral minus strand DNA with an IC50 of 0.24 µM. Other analogs had a higher IC50: ddC-TP: 6 µM, Lamivudine-TP: 10 µM, L-FddC-TP: 27 µM. Further analysis showed that L-FD4C-TP is likely to be a competitive inhibitor of dCTP incorporation. Our data demonstrate that L-FD4C is a strong inhibitor of both DHBV reverse transcriptase activity and DHBV replication *in vivo*. Long-term administration of L-FD4C should be evaluated in terms of toxicity and efficacy in clearing viral infection.

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Utilization of transgenic mice replicating high levels of hepatitis B virus for antiviral evaluation of lamivudine

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The functionality of the hepatitis B virus (HBV) life-cycle in recently generated transgenic mice (Guidotti, et al., J. Virol. 69:6158) creates the opportunity to evaluate chemotherapeutic agents on HBV replication in a small animal model. The objective was to further validate this HBV-transgenic mouse model by comparing antiviral effects of a nucleoside analogue, lamivudine ((-)2'-deoxy-3'thiacytidine, 3TC) known to be efficacious in HBV-infected human patients, with another, ineffective nucleoside analogue (AZT, zidovudine). Male or female HBV-transgenic mice were treated *per os* twice a day for 21 days with varying dosages of lamivudine or with *ad libidum* AZT in the drinking water. Serum HBV DNA and viral antigens were monitored from weekly tail bleeding during treatment and 3 weeks after the final treatment. The results were as follows: 1) As predicted from human studies, treatments of lamivudine (100, 50 and 25 mg/kg/day) were efficacious ($P<0.01$) in reducing serum HBV DNA titers, whereas AZT was not efficacious. 2) Moreover, within one week after the last treatment of lamivudine, the viral titers rebounded to at least pre-treatment levels which is reminiscent of human results. 3) Female and male mice were both comparable and suitable for chemotherapeutic studies using serum HBV DNA and HBsAg parameters. These results further validate the usefulness of HBV-transgenic mice as a chemotherapeutic model for human infection.

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The Role of Mitochondrial Deoxyribonucleoside Kinases for the Toxicity of Nucleoside Analogs

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Nucleoside analogs are commonly used in anti-viral and anti-cancer chemotherapy. The nucleoside analogs act as DNA chain terminators or enzyme inhibitors after intracellular conversion to their corresponding 5'-triphosphate form. The rate limiting step in the activation of nucleoside analogs is regarded to be the phosphorylation of the inactive prodrug by a nucleoside kinase. The four different human nucleoside kinases are now cloned and recent studies in our laboratory have focused on their intracellular location. The S-phase specific enzyme thymidine kinase 1 is located in the cytosol. One of the constituitively expressed enzymes, deoxycytidine kinase, is imported into the cell nucleus whereas thymidine kinase 2 and deoxyguanosine kinase are expressed in the mitochondria. The constituitively expressed nucleoside kinases are all sequence related as well as related to the herpes virus thymidine kinases. Mitochondrial toxicity is known for several anti-viral nucleoside analogs. Damage of mitochondrial DNA synthesis by incorporation of nucleoside analog triphosphates has been demonstrated for AZT and FIAU. We have investigated the contribution of mitochondrial nucleoside kinases for the toxicity of nucleoside analogs. The importance of these enzymes for toxic effects by interfering with nuclear and/or mitochondrial DNA synthesis will be discussed.

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Pharmacokinetics of β-D-2',3'-Didehydro-2',3'-dideoxy-5-fluorocytidine (D-D4FC) in Rhesus Monkeys.

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β-D-2',3'-Didehydro-2',3'-dideoxy-5-fluorocytidine (D-D4FC) has potent and selective anti-HIV and HBV activity *in vitro*. The single-dose pharmacokinetic parameters of D-D4FC in rhesus monkeys after intravenous and oral administration of 33.3 mg/kg were determined using a two compartment model. Due to the lability of D-D4FC in acid, $NaHCO_3$ buffer solution was used for oral administration. The average value for the terminal half-life, $t_{1/2\beta}$, was 3.2 hr (CV = 7.4%). Average values for renal clearance (Cl_{renal}) and for total systemic clearance (Cl_{sys}) were 0.31 (CV = 13.4%) and 0.41 (CV = 4.7%) $l \cdot kg^{-1} \cdot hr^{-1}$, respectively. Oral bioavailability of D-D4FC was incomplete, with an average of 47.6% (CV = 32.5%) of the dose reaching the systemic circulation. More than 74% of the compound was recovered in the urine in 8 hr, indicating that D-D4FC was eliminated mainly by renal excretion. D-D4FC was detected in CSF at similar concentrations for both i.v. and oral routes. The favorable pharmacological profile of D-D4FC warrants its development as an antiviral agent.

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In Vivo Activity Of An Antisense Oligonucleotide Targeted Against The E1 Region Of Human Papillomavirus

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We have studied the inhibitory activity of antisense oligodeoxyribonucleotides (ODNs) that target expression of the E1 helicase region of human papillomavirus (HPV) types 6 and 11. Activity of antisense ODNs *in vitro* was measured using mammalian cells transfected with an E1-luciferase reporter gene. Selected compounds were further examined *in vivo* using a kidney xenograft model in which HPV-infected foreskin fragments are implanted under the kidney capsule of a nude mouse. A phosphorothioate 2'-O-methyl RNA hybrid (changes at the 5' end) ODN targeting the E1 AUG of HPV (known as HPV1 0x5 OMe) had good inhibitory activity in the xenograft model and reduced HPV induced condyloma growth by 95%. A mismatched ODN in which the some of the guanine bases were replaced by adenine was inactive in this model. Further studies of the activity of these ODNs was made using the mCMV infection model in mice. HPV1 0x5 OMe, but not the mismatched ODN, surprisingly protected the mice from the lethal effects of mCMV. To determine the mechanism of action of HPV1 0x5 OMe we tested this compound against another human papilloma virus - HPV40 - which did not have the same sense sequence as HPV6 and 11, and was therefore mismatched to the compound. HPV1 0x5 OMe inhibited condyloma growth induced by both HPV11 and HPV40 to the same degree. We believe that the action of HPV1 0x5 OMe in the xenograft model is not totally related to an antisense mechanism, one possible mechanism is via an immune stimulatory effect.

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Detection of Soluble Nuclear Matrix Protein (NMP) Released from Apoptotic Nuclei of Human Papillomavirus (HPV) Positive Cell Lines Treated with Cidofovir

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Acyclic nucleoside phosphonates (ANPs), in particular cidofovir, inhibit the growth of rapidly proliferating cells. This phenomenon is mainly observed in HPV-, adenovirus- and SV40-transformed cell lines. We have shown by a cellular DNA fragmentation ELISA and by cell cycle analysis that the process leading to cell death following treatment with ANPs is due to apoptosis (programmed cell death). Apoptosis is associated with changes in several cellular processes. During apoptosis, dense chromatin masses increase in number until the nucleus becomes pyknotic. The skeletal structure for the topological organization of chromatin in the interphase nucleus is NMP, and breakdown of the overall nuclear structure results in the disruption of normal chromosomal nuclear matrix interactions. We have used a sensitive NMP ELISA that demonstrates the release of NMP in a soluble form from the apoptotic nucleus. A direct relationship between the amount of detectable soluble NMP and the number of dead cells was observed when CK-1 cells (HPV-33$^+$) and HeLa cells (HPV-18$^+$) were treated with different concentrations of cidofovir and cytarabine (AraC). Thus, appearance of detectable soluble NMP, one of the products of the segmentation of the nucleus, confirms that the process leading to cell death following treatment with cidofovir is apoptosis.

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A Double-Blind, Placebo-Controlled Study of Cidofovir Gel for Human Papillomavirus (HPV)-Associated Genital Warts. R. SNOECK,[1*] M. BOSSENS,[2] D. PARENT,[3] B. DELAERE,[4] H. DE GREEF,[5] E. DE CLERCQ,[1] S. SAFRIN,[6] B. McGUIRE,[6] H. S. JAFFE.[6] [1]Rega Institute, Leuven Belgium; [2]Association Hospitaliére Etterbeek-Ixelles, Bruxelles; [3]Erasme Hospital, Bruxelles; [4]St. Luc Hospital, Bruxelles; [5]U.Z. St. Rafaël, Leuven; [6]Gilead Sciences, Inc., Foster City CA.

Cidofovir is an acyclic nucleotide analog with anti-HPV activity in animal models and anti-proliferative effect in HPV-infected cell lines. Thirty-one immuncompetent patients (18 women, 13 men) with biopsy-proven HPV-associated anogenital warts (untreated, refractory, or recurrent) were stratified by total wart area and randomized to receive 1% cidofovir (CDV) or placebo gel in a 2:1 ratio, applied qd x 5 days every other week for a maximum of 6 cycles (12 wks). Wart response at week 12 was categorized as: complete (100% clearance), partial (≥50% area decrease), no change (25-50% decrease), and progression (>25% increase). Previous therapy for warts included podofilox (37%), cryotherapy/electrocautery (34%), laser (20%), other (excision, 5-FU;17%); 57% of pts had no prior treatment. Baseline median wart surface area was 56 mm² (range,8-1756 mm²); median wart number was 8.5 (range, 1-20). Nine of 19 CDV pts (47%) had complete clearance vs. none of 11 (0%) placebo pts (p=.006). Sixteen CDV pts (80%) had a complete or partial response vs. 2 (18%) placebo pts (p=.001). Only 1 of 8 complete responders followed to date (median follow-up, 180 days; range, 127-252 days) had recurrence of same-site warts. Reversible application site reactions (pain, pruritus, ulceration, rash) occurred in 65% CDV vs. 55% placebo pts (p=0.7). No evidence of systemic toxicity has been seen.

Oral Session IV: Retrovirus Infections II

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CXCR4 is the target for the potent anti-HIV activity of the bicyclams.
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AMD3100, 1,1'-[1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane, is the prototype compound of the bicyclams and has been shown to interact specifically with the CXC-chemokine receptor CXCR4, the main coreceptor used by T-tropic HIV strains to enter their target cells. AMD3100 dose-dependently inhibited (i) the binding of a specific CXCR4 mAb to lymphocytes and T cell lines, (ii) the Ca^{2+} flux in lymphocytic SUP-T1 and monocytic THP-1 cells induced by stromal cell-derived factor 1α (SDF-1α), the natural ligand for CXCR4, and (iii) the chemotactic responses of THP-1 cells induced by SDF-1α. When certain metals such as Zn, Ni, Cu, Co and Pd were incorporated in the AMD3100 molecule the anti-HIV activity of the resulting complexes decreased in the order Zn > Ni > Cu > Co > Pd. Their anti-HIV activity was directly correlated with their potency in inhibiting the binding of the anti-CXCR4 mAb and in inhibiting the Ca^{2+} flux induced by SDF-1α. Also, the nature of the linker between the two bicyclams units is important for anti-HIV activity. For example, AMD2763, with a propylene bridge between the two cyclams units instead of phenylenebis(methylene) bridge proved about 20-fold less inhibitory to HIV-1 than AMD3100. Again, this decrease in anti-HIV activity correlated with its weaker inhibitory effect on the binding of the anti-CXCR4 mAb and on the SDF-1α-induced Ca^{2+} flux. Finally, bicyclams have no effect on the Ca^{2+} flux induced by the chemokines MIP-1α, MIP-1β, RANTES, MCP-3, IL-8 and GCP-2 (ligands for CCR-1, -2, -3 -4, -5 and CXCR-1 and -2). In conclusion, our experiments point to CXCR4 as the main target for the anti-HIV activity of the bicyclams.

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The phosphoramidate approach to the intracellular delivery of free nucleotides can enhance can potency and antiviral selectivity of a variety of nucleosides.
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We note that the phosphoramidate delivery method is successful in enhancing the antiviral potency of a range of nucleoside analogues, and appears to operate via the intracellular delivery of free nucleotides. Examples where potencies are markedly increased include d4T, ddA, and d4A. In some cases, such as ddC we noted only a retention of antiviral potency, and in other cases, the activity of the nucleoside was markedly decreased on phosphoramidate synthesis. Most notably, the anti-herpetic agent BVDU became very poorly active upon addition of the usual phenyl methoxy alaninyl phosphate unit. The anti-neoplastic drug FUDR shared the same fate; becoming poorly active on phosphoramidate formation. Similarly, small modifications in the structure of the phosphoramidate unit lead to significant changes in efficacy.
To attempt to rationalise these marked differences we have subjected each of these compounds to enzyme mediated degradation. We note that some cases of poor activity do correlate with low esterase-mediated deesterification, whilst in other cases the deesterification proceeds well, but the suggested second stage of metabolism, namely the loss of the phenyl (phosphate) group is impeded. The results lead to a useful enzyme model for the prediction of likely antiviral efficacy for phosphoramidate compounds.

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Antiviral activity of a new class of HIV protease inhibitor: Distinct resistance and protein binding properties and bioavailability. J. Saunders, A. Heldsinger, B. Neorr, T. McQuade, S. Gracheck, S. VanderRoest and L. Sharmeen. Parke-Davis Pharmaceutical Research, Division of Warner Lambert Company, Ann Arbor, Michigan

Inhibitors which target HIV protease, an essential enzyme of the human immunodeficiency virus, are an essential component of current anti-HIV therapies. However, as with all anti-retroviral therapies, the clinical benefit of protease inhibitors are often overcome by emergence of multiple mutations which exhibit cross resistance to other peptido-mimetic inhibitors. PD178390 is an example of a new and distinct class of 5,6-dihydro-4 hydroxy-2 pyrones with remarkable antiviral properties. The class retains activity against several protease resistant viruses with mutations in the following amino acids: G48V, M46I, V82T, V84I and D30N. The EC_{90} values for PD178390 against laboratory strains of HIV, clinical isolates, and protease resistant HIV, in T cell lines, PBMCs, and macrophages, range from 0.8-1.2μM. PD178390 exhibits potent antiviral activity in chronically infected cells and specifically blocks proteolysis of HIV-gag and gag-pol proteins. Continuous passage of HIV to members of this class did not select for common mutations in the protease gene. A 3-4 fold increase in EC_{50} values are observed in passaged virus with substitutions in position 10 and 36. Addition of human serum or α-acid glycoprotein (AAG) resulted in 2-4 fold changes in the EC_{50} values. Bioavailability in mice suggest that adequate plasma levels will be achievable to produce an antiviral effect. For example, mice dosed orally with PD 178390 at 25 mg/kg had a Cmax of 26μM, $T_{1/2}$ of 2.2 hours, and the drug plasma concentration exceeded the EC_{90} values for about 8 hours. The bioavailability, comparing PO and SC dosing, was 63.1% for this compound. The antiviral properties of PD178390 against resistant viruses and its bioavailabilty demonstrates the potential clinical utility of this compound.

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Anti-HIV Activity of Protease Inhibitors in Productively-Infected Macrophages, and Correlation with Clinical Outcome.
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Protease inhibitors (PIs) act at late stages of replication of human immunodeficiency virus, and thus should be able to inhibit HIV replication also in chronically-infected cells (a relevant viral reservoir in the body). To assess this hypothesis, human primary macrophages already infected by HIV were treated with saquinavir, ritonavir, or KNI-272 (three different PIs). The antiviral activity was compared with that achieved in other cell types. All three PIs substantially inhibited the replication of HIV in macrophages already carrying proviral genome at the time of treatment, yet this effect has been achieved at concentrations 21-60 fold greater than those active in acutely-infected lymphocytes, 8-24 fold greater than those in acutely-infected macrophages, and 2-6 fold greater than those in chronically-infected T-lymphocytes. Such effect has been confirmed by western blot analysis, showing the appearance of immature gag proteins in supernatants and cell lysates of macrophages only at micromolar concentrations. Moreover, complete inhibition of virus production was achieved in all other cell types, but not in chronically-infected macrophages at the non-toxic concentrations used in our experiments. Finally, the removal of drugs from culture rapidly brought virus production back to the levels detected in untreated macrophages. A genetic analysis of the protease of monocytotropic strains shows an overlapping sequence with that of lymphocytotropic strains, thus suggesting that the lower antiviral effect of PIs in chronically-infected macrophages is not related to an intrinsic resistance of the strains used in our experiments.
PIs are thus active *in vitro* in chronically-infected macrophages at micromolar concentrations (EC50s between 0.7 and 4.8uM), that is in the upper range of the concentrations achieved in the plasma of treated patients. For this reason, chronically-infected macrophages may be able to produce virus in case of inconsistent treatment, lack of compliance, and/or higher drug metabolism, thus contributing to drug failure or viral breakthrough occurring in patients after prolonged therapy.

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Blocking the entry of T-cell- and macrophage-tropic HIV-1 strains by targeting a common post-CD4-binding step. KJ. Sastry[1,2], PN. Nehete[1], AK. Sarkar[1], J. Liu [2], & RB. Arlinghaus[2], Depts [1]Vet. Sci & [2]Mol. Path., The U.T.M.D.Anderson Cancer Center, [1]Bastrop, & [2]Houston, TX

The T-cell- and macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1) infect $CD4^+$ cells that also express co-receptor molecules belonging to the CXC and/or CC subfamily of the seven transmembrane domain G protein-coupled receptors. While the exact sequence in the HIV envelope protein gp120 that interacts with these co-receptors is not known, the V3-loop region of gp120 has been implicated as an important determinant for the cellular tropism of phenotypically different viral strains. We obtained evidence to show that the central portion of the V3-loop region comprising of 15-21 amino acids is important for HIV infection, because synthetic peptides corresponding to this sequence from either T-cell- or macrophage-tropic HIV strains blocked the entry of both types of HIV isolates into $CD4^+$ cells. Also, the V3 peptides efficiently inhibited HIV-induced syncytium formation. These HIV-inhibitory V3 sequences specifically bind to target cells, and the binding is competed by purified viral particles, but not by rgp120, sCD4, or β-chemokines. Additionally, the V3 peptides did not block rgp120-sCD4 interaction nor alter the cell surface CD4 expression, but inhibited gp120-induced ternary-complex formation between CXCR4, CD4 and gp120. Together, these results indicate that the V3-loop region of gp120 interacts with host cell surface during HIV infection at a post-CD4-binding stage, but prior to the co-receptor interaction, that is common and critical to both T-cell- and macrophage-tropic HIV strains. These results combined with exteded physical and biological stability observed for these V3 synthetic peptides in human plasma/serum, strongly suggest their utility for developing novel HIV therapeutics.

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Anti-HIV Activity in the HuPBMC SCID Mouse Model of Six Novel Nucleoside Analogs: (-)-FTC, (+/-)-FTC, D-DAPD, D-D4FC, CS-92 and CS-87

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Six clinical candidates have been tested for their ability to inhibit HIV replication in SCID mice reconstituted with human peripheral blood mononuclear cells (HuPBMC SCID mice). Two oxathiolane nucleosides, (-)-FTC and the racemic mixture (+/-)-FTC; the dioxolane nucleoside D-DAPD; D-D4FC; CS-92 and CS-87 were administered i.p. twice a day in doses of 60 mg/kg per day from day -1 to day +6 post infection. (-)-FTC completely prevented infection of all mice but was only slightly more effective than (+/-)-FTC (as measured by quantitative coculture). D-DAPD and D-D4FC reduced levels of infectious virus in peritoneal cells, lymph nodes, spleen and blood. CS-92 and CS-87 were also effective. Effects on intracellular and plasma viral RNA copies as measured by NASBA will be reported. FACS analysis revealed protection of human CD4 cells from HIV-induced destruction. Drugs that were most effective at inhibiting HIV replication tended to provide the greatest protection of CD4 cells. These compounds warrant further development as antiviral agents against HIV infection.
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Preclinical Pharmacokinetic Evaluation in Rat Brain and Plasma of Selected Urea-PETT Compounds

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The optimization process for the urea-PETT compounds has led to the PETT-4 compounds. A pharmacokinetic evaluation in rats of two compounds, MSH-372 and MSC-206 (a prodrug of MSC-204) showed that oral administration of MSH-372 at a dose of 10 mg/kg (0.027 mmol/kg) gave plasma levels in the micromolar range with a C_{max} of 0.62 µM at 8 hrs suggesting once per day dosing is feasible. Oral dosing (0.053 mmol/kg) of the more soluble prodrug MSC-206 also gave plasma levels of the active compound, MSC-204, in the micromolar range with a C_{max} of 1.12 µM at 3 hrs.. MSH-372 and MSC-206 were given iv at doses of 11 and 18 mg/kg (0.031 mmol/kg) respectively and the levels of MSH-372 and MSC-204 in plasma and brain tissue at 15, 60 and 120 min were determined. The concentrations in brain were for MSH-372; 4, 2 and 1 µg/g brain tissue and for MSC-204; 2, 2 and 1.5 µg/g brain tissue, which corresponds well with the corresponding plasma levels. These results clearly show that high levels of the PETT-4 compounds could be detected in the brain, which is an important property for drugs directed against HIV/AIDS.

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Predictors of Clinical Progression and Adverse Events in a Cohort of HIV-infected Patients with Advanced Immunodeficiency Started on Protease Inhibitors. F. RAFFI, G. CHENE, I. ARNAUD, L. DEQUAE, C. CHIDIAC, J.P. COULAUD, P. DELLAMONICA, J. FROTTIER, C. KATLAMA, J.Y. LACUT, P.MASSIP, T. MAY, E. PICHARD, J.M. RAGNAUD, J.L. SCHMIT, F. VACHON, C. LEPORT. Univ. Hosp. Nantes, INSERM U330, Bordeaux, APPIT, Paris, France.

A prospective multicentre cohort of 451 patients (pts) starting protease inhibitors (PI), in 13 AIDS centers, was designed to identify prognostic factors associated with AIDS-related clinical events and adverse events (AE) in HIV-infected pts with advanced immunodeficiency started on PI. Clinical and therapeutic data, CD4+ cell counts, plasma HIV RNA were collected at day 0 of PI, Month (M) 1, M3 and every 3 M thereafter. Cox models were used to estimate the independent effect of potential predictive factors. At entry, 63% pts had AIDS, median CD4 cell count = 22/mm3 (range: 0-444), mean plasma HIV RNA = 5.1 $\log_{10}$ copies/ml (SD: 0.7), mean previous antiretroviral therapy = 33 M (92% non naive). The PI used were ritonavir (n=199), indinavir (n=149) and saquinavir (n=103). At M3, the incidence of AIDS events was 9.5% and the incidence of AE was 28.5%. In the multivariate analysis, short-term occurrence of AIDS events was associated with AIDS prior to PI (RR=5.0 vs non AIDS, $p<0.01$), CD4 count <20/mm3 (RR=2.0 vs CD4 ≥20, $p=0.04$), saquinavir (RR=2.0 vs indinavir, $p=0.04$) or ritonavir (RR=2.9 vs indinavir, $p=0.01$). Occurrence of any AE was associated with AIDS prior to PI (RR=1.7 vs non AIDS, $p=0.01$), CD4 count <20/mm3 (RR=2.2 vs CD4 ≥20, $p<0.01$), indinavir (RR=2.4 vs saquinavir, $p<0.01$) or ritonavir (RR=2.6 vs saquinavir, $p<0.01$). PI interruption within the first 3 M was more frequent with saquinavir (31%) or ritonavir (31%) than indinavir (8%), $p<0.01$. The analysis of updated follow-up at 12 M is under way.
In these pts with advanced immunodeficiency started on PI, different factors were associated with short-term occurrence of AIDS events or AE. Clinicians may use these prognostic factors for guidelines to monitor pts started on PI.

Poster Session I: Retrovirus, Hepadnavirus, Papillomavirus Infections

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Evaluation of Inhibitors of Chemokine Receptor Binding in a Cell-Based ELISA. Lackman-Smith, C.S., Halliday, S.H. and Buckheit, R.W. Jr. Southern Research Institute, Frederick, MD, USA

The development of monoclonal antibodies targeted to chemokine binding sites, such as CXCR4 and CCR5 on HIV-infectable cells has led to an increased understanding of the mechanisms of both virus entry and cellular tropism, as well as the nature of the activity of compounds which inhibit early events in HIV infection. We have implemented a versatile cell-based enzyme linked immunosorbent assay (ELISA) which exploits the interaction between the chemokine receptors and the available monoclonal antibodies targeted to those receptors. Samples are assayed in a 96-well plate format with colorimetric or fluorimetric endpoint detection, depending on the secondary antibody selected. This assay is a modification of staining procedures utilized for flow cytometry and, as such, minimizes handling of individual samples in tubes as well as the time required for data acquisition and analysis. Presently, approximately 60 compounds can be tested in duplicate in the amount of time required to evaluate one compound by flow cytometric methodology. Data will be presented on the screening of several compounds in this medium throughput assay system.

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Antisense oligodeoxynucleotide inhibits HIV-1 second receptor : chemokine CC-CKR5 in macrophage-tropic COS cell line

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The chemokine receptor CC-CKR5 (CKR5) was recently demonstrated to be a co-receptor for macrophage-tropic HIV-1 strain(1). In the present study, we report the effects of antisense oligonucleotides on CKR5 production on the macrophage-tropic COS cells, to find more efficacious therapeutic possibilities for treatment of HIV-1.

Several sequences of phosphodiester(O-) and/or phosphorothioate(S-) oligonucleotides complementary to bases-(223-242) of the CKR5 mRNA gene were synthesized. Human COS cells with stable expressing human CD4 and CKR5 were grown in DMEM containing 10% heat-inactivated fetal calf serum. The total p24 concentration depends on HIV-1 infection was detected using an enzyme-linked immunosorbent assay (ELISA) system.

The antisense S-oligos, complementary to the mRNA gene including initiation codon of the CKR5, inhibited the p24 production in a dose-dependent manner. Further, antisense O-oligos complexed with cationic-and hydrophilic random copolymer viz., poly-l-lysin / serine, inhibited p24 production at lower concentartion. On the other hand, sense O-oligos complexed with said polymer had no effects.

Human CKR5 antisense oligonucleotides inhibited the HIV-1 infection of COS cells, suggesting a potential to reduce some kinds of inflammatory processes.

Reference

(1) Ghalib Alkhatib, et al., SCIENCE 272,1955-1958(1996).

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SIVmnd Uses CXCR4 as Coreceptor for Entry in Human Cells

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It has been clearly demonstrated that SIV and M-tropic HIV-1 strains use CCR5 as a coreceptor for entry in human cells and that CXCR4 is the main coreceptor for T-tropic HIV-1 and HIV-2 strains. For SIV, coreceptor use studies have been generally done with SIVmac (macaque), and occasionally also with other SIV strains such as SIVagm (african green monkey), SIVcpz (chimpanzee) and SIVsm (sooty mangabey). It was uniformly concluded that SIV uses CCR5 (or other coreceptors), but not CXCR4. Four distinct groups of lentiviruses have been identified: HIV-1, SIVsm/SIVmac/ HIV-2, SIVagm and SIVmnd (mandrill). Here we demonstrate that SIVmnd uses CXCR4 to enter human cells. SIVmnd is inhibited by the stromal cell-derived factor 1α (SDF-1α), the natural ligand for CXCR4. The IC_{50} is 75 ng/ml, which corresponds to the IC_{50} of SDF-1α for T-tropic HIV-1 strains. A specific anti-CXCR4 mAb inhibits the replication of the SIVmnd strain at an IC_{50} of 1 μg/ml. Also the IC_{50} (8 ng/ml) of the bicyclam AMD3100, a specific CXCR4 antagonist, is comparable with its IC_{50} for T-tropic HIV-1 and HIV-2 strains. The SIVmnd strain replicates only in HOS.CD4 cells expressing CXCR4 and not in HOS.CD4 cells expressing CCR1, CCR2b, CCR3, CCR4, or even CCR5. This is the first SIV strain described so far to use CXCR4, and not CCR5, as a main coreceptor for entering human T cells.

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AMINO ACID PHOSPHORAMIDATES OF ANTIVIRAL 3-DEAZA-ADENOSINE ANALOGS EXHIBIT HIGH POTENCY AND LOW TOXICITY.

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Due to the debilitating and ultimately fatal nature of AIDS, an intense effort has been underway to improve existing treatments and to develop new therapies. Currently, nucleoside based reverse transcriptase inhibitors are being successfully employed as potent antiviral agents. Although the mechanism of action is unknown, the 3-deazaadenosine analogs, 3-deaza-adenosine (DZA), 3-deaza-(±)-aristeromycin (DZAri), 3-deazaneplanocin A (DZNep) have been found to have significant anti-HIV activity against AZT sensitive and resistant clinical isolates of HIV-1. Unfortunately, these compounds are at least ten fold more toxic to PBMCs than AZT. Recently, we have demonstrated that amino acid phosphoramidates of AZT and d4T exhibit significant antiviral activity with considerably reduced cytotoxicity. Consequently, in order to examine the generality of this approach, we have constructed phenylalanine and trytophan phosphoramidates of DZA, DZAri and DZAra-A. Preliminary results have demonstrated that the tryptophan phosphoramidate is nearly forty fold more active than DZA, while the phenyl-alanine derivative is nearly ten fold more active than DZA. As was observed for AZT-phosphoramidates, the DZA phosphor-amidates exhibited no detectable cytotoxicity to PBMCs at concentrations as high as 10 micromolar, while the CC_{80} for DZA is one micromolar. Surprisingly, unlike DZA, the phos-phoramidates of DZA did not exhibit activity against AZT resistant HIV-1. Experiments are currently underway in order to address the possibility of a unique mechanistic rationale for the activiral activity of these compounds.

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ANTI-HIV ACTIVITY OF NEW COMPOUND : PRODRUG OF D4T

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D4T, a thymidine analog with potent anti-HIV activity in-vitro, is currently investigated as therapy for patients with advanced HIV infection. The limitant step of this dideoxynucleoside analog is its conversion to 5'-monophosphate. In an effort to overcome this restriction, the prodrug derivative of d4T has been synthesized and evaluated for its inhibitory effects on the replication of HIV-1 in human T4-lymphoblastoid cell lines and CEM cells as well as in thymidine kinase deficient (TK^-) CEM cells.
The method of preparation of this new prodrug of d4T is relatively simple to use. It can be obtained in two steps starting from d4T with 56% yield after purification. The first step consisted with the preparation of intermediate which is activated in the second step to react in-situ with d4T.
This new compound has been evaluated for its inhibitory effects on the replication of HIV-1 in various cells culture systems. It proved to be more effective than d4T in inhibitoring HIV-1 replication in wild type MT4 cells and as efficient as d4T in thymidine kinase positive (TK^+) CEM cells.
Moreover, as expected, d4T proved to be weakly active against HIV-1 replication in thymidine kinase deficient (TK^-) CEM cells with a 50% effective concentration $(EC)_{50}$ value at 10 μM. In contrast, in CEM/TK^- cells, the corresponding prodrug of d4T emerged as a potent inhibitor with an $(EC)_{50}$ value at 0,3 μM.
This new compound is attached into the prodrug strategy and opens a wide field of studies in chemotherapy and could be a great help in the design of new anti-HIV prodrugs. Work along these lines is in progress in our laboratory.

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Acuvity of masked 2'-3'-dideoxynucleoside-monophosphate derivatives against human immunodeficiency virus in resting macrophages

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Resting cells are characterized by low activity of the kinases that catalyze the first phosphorylation step of nucleoside analogues. For this reason, the direct delivery within resting cells of monophosphate-derivatives of nucleoside analogues may afford greater levels of phosphorylation and, consequently, greater antiviral activity. Objective of our study was the assessment of the anti-HIV activity of arylphosphoramidate derivatives of d4T, AZT, ddA, d4A, ddC and 3TC in macrophages (resting cells characterized by continuous high levels of virus production), and the comparison with the antiviral activity of the parent nucleosides. So324 (the d4T-MP prodrug) was quite potent in macrophages, with activity about 25 fold greater than that of d4T in the same cells (EC50: 0.008 μM and 0.20 μM, respectively). Even more evident, Cf1001 (the d4A-MP prodrug) was characterized by an EC50 of 0.01 μM in macrophages, while the parent compound was almost devoided of any activity. Also Cf1093 (the ddA-MP prodrug) showed an EC50 of 0.005 μM, compared to 1 μM for the parent compound ddA. By contrast, So221 (the AZT-MP prodrug) was 6 fold less active in macrophages than AZT (EC50: 0.012 μM and 0.002 μM, respectively). The same was found for the ddC-derivative (Cf1221) that was still 10 fold less active than ddC in macrophages (EC50: 0.09 μM and 0.001 μM, respectively), while the activity of 3TC-derivative was similar to that of the 3TC in these cells. In general, a greater activity of all nucleotide prodrugs tested has been detected in macrophages compared to lymphocytes, and this is in agreement with previously published data. Nevertheless, not all aryl-phosphoramidate substitutions let to an increased antiviral activity compared to parent compounds in macrophages. This is somewhat related to the dynamics of phosphorylation of each nucleoside (that is, AZT has its bottleneck at the level of the second phosphorylation, and thus is not bypassed by AZT-MP phosphoramidate, while the bottleneck of d4T is at the first phosphorylation step). The selective delivery of phosphorylated compounds is relevant from the clinical standpoint, due to the need to reach in tissue macrophages drug levels sufficiently high to overcome viral replication, and thus to affect the outcome of the disease in all body compartments (including the central nervous system) where HIV hides and replicates.

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Studies Into The Role of The 5'-*tert*-Butyldimethylsilyl Group In The Anti-HIV-1 Activity of TSAO Derivatives

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Among the different families of non-nucleoside reverse transcriptase inhibitors (NNRTI) of HIV-1 replication described so far, TSAO derivatives represent a particular and peculiar group of inhibitors. In their interaction with HIV-1 reverse transcriptase, they behave as NNRTI, while, structurally, they are highly functionalized nucleosides. In our ongoing program directed to the improvement of the antiretroviral profile of the potent and selective HIV-1 RT inhibitor TSAO-T we were interested in determining the role that the *tert*-butyldimethylsilyl groups (TBDMSi) both at position 2' and 5' of the sugar moiety may play in the interaction of TSAO derivatives with the HIV-1 RT. Structure activity relationship studies revealed that both TBDMSi groups are needed for optimal interaction of TSAO-T with the HIV-1 RT, however, the 2' TBDMSi group is less critical for activity than the 5'-TBDMSi group. Studies on the inhibition of HIV-1 RT by TSAO-T indicated there is no transfer of TBDMSi groups from TSAO-T to the enzyme. This prompted us to synthesize and test for antiviral activity various TSAO derivatives in which the 5'-TBDMSi group has been replaced by groups that mimic either steric, lipophilic or stereoelectronic properties of that group. Among the compounds prepared only those bearing allyl or methylidene substituents at the 5'-position were endowed with antiretroviral activity. However, this was at least two orders of magnitude lower than the prototype compound. Our synthetic strategies fro the preparation of these new TSAO analogues and their anti-HIV-1 activity in vitro will be presented.

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Further Insights Into The Role of The Thymine Moiety of TSAO-T in The HIV-1 RT Inhibition

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TSAO derivatives represent a peculiar group of non-nucleoside reverse transcriptase inhibitors (NNRTI) of HIV-1 replication. In their interaction with HIV-1 RT, they behave as NNRTI, while, structurally, they are highly functionalized nucleosides. So far, structure-activity relationship studies have shown that the sugar part of the TSAO molecules plays a principal and crucial role in the interaction of TSAO compounds with their target enzyme RT. However, the role of the base part in this interaction is yet unclear. The thymine moiety of TSAO-T can be replaced by other nucleobases or heterocycles without marked decrease of antiretroviral efficacy. In order to gain insight in the interaction points of the TSAO derivatives with the HIV-1 RT and in particular to determine the role that the nucleobase may play in this interaction we focused our attention on the modification of the base part of the prototype compound TSAO-T. Therefore, we prepared novel TSAO analogues that maintain the spirosugar part of TSAO-T substituted at the anomeric position with non aromatic rings and substituted urea or thiourea moieties derived from a systematic disassemblage of the molecular architecture of the thymine of the prototype compound. The new compounds prepared allowed us to asses: 1) the relevance that the aromaticity of the base may play in the interaction of the compounds with HIV-1 RT; 2) the role that different fragments mimicking parts or the whole thymine base of TSAO-T play in that interaction. Our synthetic strategies for the preparation of these new TSAO analogues and their anti-HIV-1 activity will be presented.

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A pyrido [1,2a] indole derivative identified as a novel non-nucleoside reverse transcriptase inhibitor of HIV-1

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A pyrido [1,2a] indole derivative was identified as a potent inhibitor of HIV-1 replication. Using the MTT cell viability assay, the 50% inhibitory concentration (IC_{50}) was 0.7μM±0.2μM and the 50% cytotoxic concentration (CC_{50}) was 23μM ±3μM. The compound showed no antiviral activity against HIV-2 or in cells chronically infected with HIV-1, but had good inhibitory effect against purified HIV-1 reverse transcriptase (RT) in an *in vitro* assay and was classified as a non-nucleoside RT inhibitor (NNRTI). It also had similar antiviral activity as the licensed NNRTI nevirapine, against a range of primary clinical isolates of HIV-1 cultured in PBMCs, and had equivalent antiviral activity against viruses resistant to the nucleoside RT inhibitors zidovudine, didanosine and lamivudine. Viruses resistant to a range of NNRTIs with the single L100I, K103N, V106A, E138K, Y181C and Y188C amino acid changes in the RT were also sensitive to the drug, which indicated a different pattern of resistance compared with other compounds of this class. Virus showing >10-fold resistance to this pyrido [1,2a] indole derivative was however rapidly selected for after growth in increasing concentrations of compound, and was shown to be cross-resistant to nevirapine. We will present data relating to the amino acid changes selected for after growth of HIV-1 in the presence of this compound.

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Antiviral Properties of the Nonnucleoside Reverse Transcriptase Inhibitor UC781 Buckheit, R.W., Jr., Fliakas-Boltz, V., Kinjerski, T.L., Russell, J.D., and Pallansch, L.A. Southern Research Institute, Frederick, MD, USA

The nonnucleoside reverse transcriptase inhibitor UC781 has been described as a highly potent anti-HIV-1 agent. UC781 has been determined to inhibit laboratory-derived and primary virus isolates at low nanomolar concentrations in both established and fresh human cells. In our evaluations, we have not been able to confirm the ability of UC781 to directly inactivate HIV-1 as has been reported recently. UC781 was found to be less active against viruses with the L100I, K103N and Y181C amino acid changes in the RT. The broad therapeutic index of UC781 (>62,000) resulted in effective inhibition of NNRTI-resistant virus isolates at high nanomolar concentrations. Upon *in vitro* selection, UC781 initially yielded resistant virus with the Y181C mutation in the RT. However, continued selection of resistant strains resulted in the accumulation of amino acid changes in the reverse transcriptase, yielding a virus that was nearly insensitive to UC781. This UC781-resistant virus was highly cross-resistant to all of the other UC compounds evaluated. UC781 synergistically interacted with nucleoside analogs to inhibit HIV-1 replication. Further, UC781 and the NNRTI costatolide were able to synergistically inhibit HIV-1 replication when used in combination, suggesting that UC781 may interact with the RT differently than the other UC analogs.

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Antiviral Activity Profiling of the Furopyridine-thiopyrimidine, PNU-142721: a Novel, Potent, HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitor. R.A. Olmsted[1], L.A. Kopta[1], D.G. Wishka[2], M.J. Murphy[2], S.T. Schlachter[2], R.A. Nugent[2] and J. Morris[2]. Infectious Diseases Research[1] and Medicinal Chemistry Research[2], Pharmacia & Upjohn, Kalamazoo, MI

Discovery efforts to identify compounds with broad activity against drug resistant variants of HIV-1 have led to the discovery and development of the furopyridine- thiopyrimidine class of non-nucleoside reverse transcriptase inhibitors (NNRTIs). Preclinical evaluation of this class of NNRTIs resulted in the selection of PNU-142721 as a clinical candidate to follow the recently approved BHAP NNRTI, delavirdine (DLV, PNU-90152; RESCRIPTOR). PNU-142721 demonstrated potent activity against HIV-1_{IIIB} and *in vitro* selected DLV-resistant variants of HIV-1 harboring the P236L or L100I RT substitutions (IC_{90}s: 1 nM, 8 nM and 70 nM, respectively). Importantly, PNU-142721 significantly suppressed the replication of twelve clinical isolates of HIV-1 highly resistant to DLV, zidovudine (AZT) or both RTIs obtained from patients who had received 24-36 weeks of combination therapy (Mean $IC_{90} = 0.08\ \mu M \pm 0.08$). In addition, PNU-142721 demonstrated similar or more potent activity against one DLV resistant and two DLV/AZT coresistant clinical isolates compared to two NNRTIs, DMP-266 and HBY-097, currently in clinical development. The potency of PNU-142721 also compared favorably with DMP-266 and HBY-097 against DLV resistant and wild-type laboratory strains.

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Binding of HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors to Human Serum Albumin and Human α_1Acid Glycoprotein : Conflicting Results Depending on the Assay Used. M.-P. de Béthune[1], K. Andries[2], H. Azijn[1], J. Van Peel[1] and R. Pauwels[1]. [1]Tibotec NV, Edegem, Belgium and [2]Janssen Research Foundation, Beerse, Belgium.

Binding of HIV-1 inhibitors to human serum proteins can strongly impair their antiviral activity. In order to assess the importance of this phenomenon for a particular drug, in vitro assays were designed in which the influence of human serum albumin (HSA) and human $\alpha_1$acid glycoprotein (AAG) on the anti-HIV activity of the tested compound is measured. In a first series of experiments, we have determined the IC_{50} of Loviride (R089439), Nevirapine, DMP266 and Delavirdine, in the presence of AAG (2, 1.5 or 1mg/ml) or HSA (4.3mg/ml) using the MTT method according to Pauwels et al. (1988, J. Virol . Meth **20**:309). Data showed that the presence of AAG resulted in a highly decreased anti-HIV activity for Loviride (±50 fold), whereas no significant change could be observed for the other compounds. The addition of HSA to the culture medium did not influence the antiviral activity of any of these drugs in this experimental setting. In a second series of experiments, we used the method described by Nunberg et al. (1991, J. Virol **65**:4887-4892) where virus replication is monitored by measuring p24 antigen production. Additionally, we assessed the percentage of infected cells in these experiments. The results with p24 are similar to those obtained in the first assay except for DMP266 which shows a significant decrease (± 10 fold) in activity in the presence of human serum proteins. However we could not observe any decrease in anti-HIV activity for DMP266 when monitoring the percentage of infected cells. Experiments are performed to elucidate these discrepancies.

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Pentafuside (T20), A Novel Inhibitor of HIV-1 Fusion and Infection, is Synergistic When Used in Combination with Reverse Transcriptase (RT) and Protease Inhibitors *in vitro*. <u>**Shawn Barney**</u>**, Kelly Guthrie, Diana Davis, Sam Hopkins, M. Ross Johnson and Dennis M. Lambert, Trimeris, Inc., Durham, NC 27707.**

Pentafuside, a 36-mer synthetic peptide derived from the HIV-1 gp41 transmembrane protein, is a potent and selective *in vitro* inhibitor of HIV-1 fusion (IC_{50} = 2.5 ng/ml) and infectivity (Vn/Vo = 0.1 at 250 ng/ml). Clinically the compound demonstrates a beta phase t1/2 of 2 - 2.5 hours and reduces viral load by at least 1.5 $\log_{10}$ in a phase I/II clinical trial (Saag et al., 1997 IDSA Meeting Abstract 771). T20 was tested alone and in a series of two drug combination studies with several reverse transcriptase and protease inhibitors in HIV-1 IIIB CEM infectivity assays. Computer analysis of drug-drug interactions demonstrated that AZT and T20, as well as, 3TC and T20 combinations were synergistic (CI = 0.3 - 0.7). Like-wise, synergistic interactions were observed with Indinavir and T20 (CI = 0.4 - 0.7) and with Nelfinavir and T20 (CI = 0.5 - 0.6). T20, which has a unique mechanism of action relative to existing therapies, demonstrates positive drug-drug interactions with representative RT and protease inhibitors. These results warrant further clinical evaluation of T20 in combination with other anti-retroviral therapies.

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In vitro synergy studies with MKC-442, a non-nucleoside HIV-1 reverse transcriptase inhibitor. E. Hill, N. Taylor, K. Borroto-Esoda, P. Furman, C. Moxham, and G. Painter. Triangle Pharmaceuticals, Inc. Durham, NC. USA

MKC-442, (6-benzyl-1-(ethoxymethyl)-5-isopropyl-uracil) a non-nucleoside reverse transcriptase (RT) inhibitor, is a potent inhibitor of HIV-1 replication with an *in vitro* EC_{50} value of 14nM in a XTT assay using MT2 cells. In a Phase I/II trial, MKC-442 has demonstrated significant antiviral efficacy (mean reduction from baseline, -1.41 $\log_{10}$) and a favorable safety profile as monotherapy. Recent recommended treatment strategies for patients infected with HIV-1 include triple drug combinations of nucleoside and/or non-nucleoside RT inhibitors along with a protease inhibitor. *In vitro* drug studies were carried out with MKC-442 in combination with the nucleoside RT inhibitors AZT, ddI, 3TC, Abacavir, and FTC, or with the non-nucleoside RT inhibitor Nevirapine, and with the protease inhibitors Indinavir, Saquinavir, Nelfinavir, Apranavir and DMP-450. These studies were performed using a cytoprotection assay with LAI-infected MT2 cells and XTT dye development as the endpoint measurement. Isobologram analysis of the resulting dose response curves showed that the combination of MKC-442 and Nelfinavir was strongly synergistic, all other combinations with MKC-442 were synergistic with the exception of Nevirapine which was additive to synergistic. None of the drug interactions were antagonistic. This *in vitro* data suggests that MKC-442 is synergistic when combined with other anti-HIV drugs especially the protease inhibitor Nelfinavir.

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Synthesis and SAR Studies of Urea PETT Compounds Leading to the PETT-4 Series, Highly Potent Allosteric HIV-1 RT Inhibitors.

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SAR optimization of the urea-PETT series of compounds has led to the PETT-4 compounds. Two enantiomeric pairs have been developed. The SAR work leading to the PETT-4 derivatives as well as their enantioselective syntheses will be described. The syntheses of these compounds are straightforward and the key step is a catalytic asymmetric cyclopropanation giving high enantiomeric excess. The anti-HIV-1 activity was determined in MT-4 cells using both wild type (wt) and mutant viruses (100I, 103N and 181C). Activity in the sub-nanomolar range was observed for the wt virus, which was better than that observed with other inhibitors such as nevirapine, delavirdine, HBY 097 and DMP 266.

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Anti-HIV Activity of PETT-4 *In vitro*

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Four compounds, MSH-372, MSN-034, MSC-204 and MSC-215 in the urea-PETT series of allosteric HIV-1 RT inhibitors have been evaluated against several HIV-1 isolates in different cell lines. ED_{50} values below 1 nM were observed for HIV-1_{IIIb} replication in MT4 cells as well as for the replication of clinical isolates of HIV-1 in MT2 cells and PBMC. The compounds were also tested against several HIV-1 isolates harbouring clinically important single amino acid changes such as L100I, K103N, Y181C and Y188L. MSH-372 (ED_{50}=0.4 µM) and MSN-034 (ED_{50}=0.6 µM) were 10 times more active against the K103N resistant isolate than DMP 266 (ED_{50}=3.8 µM) when measured in the presence of 50% human serum. Antiviral activities against isolates containing two amino acid changes (L100I+ K103N), (L100I+V108I) and (L100I+Y181C) were also determined and inhibition was seen at micromolar concentrations. In vitro selection of resistant virus showed that for MSC-204, mutant virus appeared after 13 weeks while it took more than 15 weeks before there were any signs of virus breakthrough in the presence of either MSH-372 or MSN-034. The development of resistance was considerably slower for these compounds than for nevirapine, delavirdine or 3TC in this model. Combinations of PETT-4 compounds with AZT, ddI, ddC, d4T or saquinavir showed additive or weakly synergistic effects.

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Antiviral Effect of PETT-4 Compounds in Monkeys Infected with SHIV.

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The PETT-4 compounds constitute a series of very potent HIV-1 inhibitors. In vitro inhibition of HIV-1 replication is seen at concentrations down to sub-nanomolar levels. The PETT-4 compounds are HIV-1 specific, like other allosteric HIV-1 reverse trancriptase (RT) inhibitors and thus in vivo evaluation could not be performed in macaques infected with SIV or HIV-2. A chimeric SIV with HIV-1 RT (SHIV) was used for *in vivo* antiviral evaluation. MSC-206 (3 x 0.5 mg/kg for 5 days), the first PETT-4 compound to be tested in this model, was given to two cynomolgus monkeys in conjunction with SHIV-inoculation (>100 monkey infectious doses i.v.) and the effect on viral antigen appearance was compared to four control animals. The appearance of both viral antigen and viral antibodies were delayed compared to the controls. MSC-206 (3 x 0.5 mg/kg) showed a better antiviral effect against SHIV in cynomolgus macaques than when 3x15 mg/kg of AZT was used. Lower doses of MSC-206 and other PETT-4 compounds are being evaluated in this model.

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Acridone derivatives are transcriptional inhibitors of human immunodeficiency virus type 1 (HIV-1)

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We have found some acridone derivatives to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) replication in chronically HIV-1-infected cells. 1-hydroxy-10-methyl-9,10-dihydroacrid-9-one (RD6-5071), the most potent compound of this series, repressed the TNF-α-induced expression of HIV-1 in OM-10.1 cells. The 50% effective concentration of RD6-5071 was 2.0 µg/ml, which was below its 50% cytotoxic concentration. RD6-5071 was also inhibitory to the expression of HIV-1 in U1 cells (another chronically infected cell line). Furthermore, RD6-5071 could also inhibit the replication of HIV-1 in U937 and peripheral blood mononuclear cells acutely infected with HIV-1. In search for its mode of action, RD6-5071 was found to be an inhibitor of protein kinase C (PKC), suggesting that the acridone derivatives inhibit the activation of HIV-1 genome through blocking a signal transduction pathway involving PKC activation.

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RD6-Y664, Benzylhydroxylamine derivative, inhibits the replication of human immunodeficiency virus.

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We have examined a novel benzylhydroxylamine derivative, RD6-Y664, for its inhibitory effects on the replication of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) in cell cultures. RD6-Y664 inhibited the replication of HIV-1_{IIIB} strain at a concentration of 1.58 μg/ml with a selectivity index greater than 39 in MT-4 cells. It also inhibited the replication of nonnucleoside reverse transcriptase inhibitor-resistant mutant (HIV-$1_{IIIB\,R}$) and HIV-2 (LAV-2_{EHO}) in MT-4 cells. This compound did not inhibit the activities of HIV-1 reverse transcriptase and protease in enzyme assay systems. The compound was not inhibitory to gp120-CD4 interaction. These results suggest that RD6-Y664 inhibits HIV replication through a novel mechanism and should be further pursued for its potential as a chemotherapeutic agent against AIDS.

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Fluoroquinoline Derivatives as Potent Inhibitors of HI Replication and Cytokine Production

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We have recently identified the fluoroquinoline derivative 12 as a potent and selective inhibitor of HIV-1 transcript In the search for more effective derivatives, we have rece found K-37 and K-38 to be more potent inhibitors of HI replication than K-12. The EC_{50}s of K-37 and K-38 were and 3.8 nM in peripheral blood mononuclear cells (PBM respectively. These values were approximately 3- and 24- lower than the EC_{50} of K-12. K-38 was also a more po inhibitor of HIV-1 replication in chronically infected c such as TNF-α-stimulated OM-10.1 cells (EC_{50}: 17 nM K-38 and 50 nM for K-12). However, K-37 and K-38 pro to be more cytotoxic than K-12 for a variety of cell line well as PBMCs. Interestingly, K-37 and K-38 could supp the production of TNF-α and IL-6 in mitogen-stimula PBMCs and the expression of ICAM-1 in TNF-α-stimula human umbilical vein endothelial cells (HUVECs) at t nontoxic concentrations. In contrast, another K-12 derivat K-42, had similar anti-HIV-1 activity and cytotoxicity p files to those of K-12, whereas K-42 did not inhibit the cy kine production and ICAM-1 expression, suggesting that 42 is a more specific inhibitor of HIV-1 transcription.

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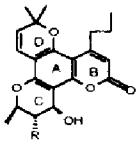
Structural Analogue of Calanolide A. Synthesis of 11-Demethyl-Calanolide A and its Anti-HIV Activities Lin Wang Chun-Mei Zhou Zhi-Zhong Zhao Xing Quan Zhang[1] Xiang-Hong Chen[1] Hong-Shan Chen[1] Institute of Materia Medica, [1] Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences. Beijing 100050, China

Recently certain coumarin derivatives, isolated from several tropical plants of the genus Calophyllum, have attracted considerable attention as potent nonnucleoside inhibitors of reverse transcriptase from human immunodefiency virus type 1(HIV-1RT). Among them(+)-calanolide A (1) is the most potent one. Our efforts focusd C ring within the (1)structure removing of C-11 methyl group in order to maitain the basic stereochemistry of (1) as well as to evaluate its anti-HIV activity. Calanolide A has three different heterocyclic rings, B,C and D constructed from a phloroglucinol core A. According to our synthetic approach, we used phloroglucinol as a starting material and then constructed the coumarin followed by chroman (C)ring. The chromene(D) ring was built last. Finally Lnche reaction reduced the chromanone to give the desired product (±)-(2). Therefore, 11-demethylcalanolide A(2) has been synthesized by a four-step reaction sequence using a simple approach. Compound (2), a white powder with melting point 150-154 ℃. Anal Cacld for $C_{21}H_{24}O_5$ C 71.33; H 7.08 Found C 70.86; H 6.91. This structure of (2) has been identified by ^{1}HNMR, MS and IR spectra. The preliminary anti-HIV tested showed that compound (2) markedly inhibited the accumulation of HIV-P24 antigen in human PBMC cell with an IC50 of 0.46 μ M.



R=CH_3, (±) calanolide A, 1
R=H, (±)-2

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Cyclic Urea and Sulfamide-based Inhibitors of HIV-1 Protease. Changes in Binding Mode upon Modifications in the P1/P1' Side Chain.

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A series of cyclic urea and cyclic sulfamide-based inhibitors directed towards the HIV-1 protease was studied with the aid of enzyme kinetics and crystallographic structure analysis. The sulfamide and urea parts of the inhibitors displace a structural water molecule which is commonly found in the protease-inhibitor complexes. Inhibition studies showed that the structure-activity relationship was different for the urea-based and the sulfamide-based inhibitors. Urea-based inhibitors were bound symmetrically, while the sulfamide-based inhibitors were bound asymmetrically. The orientation of the sulfamide-based inhibitors was found to be influenced by the identity of the P1/P1' side chain. Using derivatives of phenethyl as P1/P1' side chains resulted in 180° rotation of the inhibitor relative to the protease.

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Cyclic 7-membered Sulfamide HIV-1 Protease Inhibitors

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In recent years the importance of HIV-1 protease inhibitors in the treatment of AIDS has been well established. We have used various carbohydrates as precursors for linear HIV-1 protease inhibitors. The disclosure of DMP323[1] encouraged us to modify these into cyclic urea and cyclic sulfamide derivatives.[2] Analysis of the crystal structure of a complex between a cyclic C_2-symmetric sulfamide inhibitor and the HIV-1 protease, revealed an unexpected binding mode. One side of the inhibitor bound as expected but the other side was twisted into the reversed orientation.[3] By making unsymmetric compounds through modifications of the P2 and P2´sidechains we hope to take advatage of this unique bindig mode to find a unique resistance profile.

(1) Lam, P. et al *Science* **1994**, *263*, 380-384. (2) Hultén, J. et al *J .Med. Chem.* **1997**, *40*, 885-897. (3) Bäckbro, K. et al *J. Med. Chem.* **1997**, *40*, 898-902.

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Evaluation of the Effects of Protease Inhibitors on the Replication of Simian Immunodeficiency Viruses (SIV) *In Vitro*. R. J. Owens[1], M. C. Osterling[1], M. G. Lewis[2], R. J. Black, and R. W. Buckheit[1]. [1]Southern Research Institute, Frederick, MD, USA; [2]Henry M. Jackson Foundation, Rockville, MD, USA; [3]National Institutes of Health, DAIDS, Rockville, MD, USA

Simian immunodeficiency virus (SIV) infection in macaques is perhaps the most relevant and useful model to evaluate viral pathogenesis and antiviral vaccines and drugs for comparison with human immunodeficiency virus (HIV) infection in humans. The objective of this study was to determine whether compounds known to arrest HIV replication by specifically inhibiting viral protease activity also affected SIV replication. We compared four protease inhibitors (saquinavir, indinavir, nelfinavir, and ritonavir) against three SIV isolates (-mac251, -mac239Δnef, and PBjΔnef) grown in rhesus macaque PBMC cultures. As controls for drug inhibition of SIV replication we also compared the nucleoside analogs AZT and ddC against the three viral isolates. All four protease inhibitors were active against SIV replication, with IC_{50} values in the nM range. Saquinavir and indinavir showed the highest level of activity, with IC_{50} values similar to AZT (1-10 nM), while nelfinavir and ritonavir were 5-10 fold less active and comparable to ddC. SIVmac251 was the most sensitive strain grown in the presence of these compounds, and to a lesser extent, SIV-PBjΔnef and SIVmac239Δnef respectively. In summary, these results demonstrate that like HIV, SIV replication can also be inhibited by these specific serine protease inhibitors. Studies are currently underway to determine whether drug resistant SIV variants can be isolated in the presence of these compounds, as well as evaluation of protease inhibitors *in vivo* using the SIV/macaque model.

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Efficacy of 9-(2-Phosphonylmethoxypropyl) Adenine for Therapy of Acute Feline Immunodeficiency Virus Infection.
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To determine the efficacy of 9-(2-phosphonomethoxypropyl)adenine (PMPA) against acute and established chronic feline immunodeficiency virus (FIV) infection, cats were treated with PMPA (30 mg/kg/day subcutaneously or 60 mg/kg/day orally) vs placebo either: (1) beginning at the time of challenge with either 1 or 10 cat infectious doses (CID) of FIV isolate FIV-B-2542; or (2) beginning 8 weeks after infection with FIV-B-2542 when infection was well established. All cats that received parenteral PMPA at the time of infection remained free of evidence of FIV infection (as determined by dilutional blood mononuclear cell coculture, polymerase chain reaction, and antibody assays) in the absence of hematologic or other toxicity. By contrast, 100% of placebo-treated cats challenged with 10 CID of FIV became persistently infected and developed CD4 T cell depletion and symptoms of immunodeficiency. Oral PMPA therapy, although less effective than subcutaneous administration, still produced a 40% level of protection. PMPA therapy in cats with established infection reduced circulating viral RNA titer but not PBMC-associated, coculture-detected virus burden. In summary, prophylactic PMPA treatment blocked FIV infection and prevented FIV-related disease and short term PMPA therapy reduced active virus burden in animals with established FIV infection. PMPA provided superior antiretroviral with substantially less toxicity than either AZT or 9-(2-phosphonomethoxyethyl)adenine (PMEA) in the FIV model.

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Curcumin-like derivatives with potent activity against HIV-1 integrase: synthesis, biological evaluation and molecular modeling

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Various derivatives related to curcumin were synthesized and tested in enzyme assays as inhibitors of HIV-1 integrase (IN). Their peculiar chemical feature is the presence of cinnamoyl moieties, either free or masked, shared by numerous natural and synthetic anti-IN agents. No matter whether compounds carried one (asymmetrical analogues) or two symmetrical cinnamoyl moieties, potent anti-IN activity depended on the presence of catechol groups. In symmetrical compounds, the activity remained unmodified following shortening (because of the overlap of the CO residues of cinnamoyl moieties) or increasing (because of the introduction of a phenyl as a spacer between the cinnamoyl moieties) of the distance between the opposite ortho-bis-hydroxyphenyl groups, as well as following overlapping of the two cinnamoyl moieties and incorporation of the CO in a cycloalkanone containing a heteroatom. While active against both 3'-processing and strand transfer reactions at concentrations as low as 0.2 μM, the new derivatives, curcumin included, failed to inhibit the HIV-1 multiplication in acutely infected MT-4 cells. Nevertheless, being totally inactive against HIV-1 reverse transcriptase and cellular polymerases they could be considered specific inhibitors of IN. On the other hand, title compounds were endowed with a remarkable antiproliferative activity, whose potency correlates neither with the presence of catechol groups nor with inhibition of topoisomerases. Molecular modeling performed in order to formulate a pharmacophore hypothesis led to establish that, contrary to quercetin, cyclovalone analogs of curcumin bind to IN with the carbonyl function oriented in "sin" with respect to the vinilyc double bond assuming an extended coplanar conformation. The presence of an accessory binding site near to that complementary to the pharmacophoric cinnamoyl frame is predicted in order to explain the activity of (bis-cinnamoyl)phenyl derivatives.

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Antiretroviral Activity of TCN-P in HIV-infected Human Macrophages.

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Triciribine-monophosphate (TCN-P) is an adenosine analog. It's activity has been reported against HIV-infected established cell lines (Kucera *et al.*, AIDS Research and Human Retroviruses, Vol. 9, No. 4, 1993). In this study, we have explored the antiretroviral activity of TCN-P in HIV-infected human macrophages. Monocyte derived macrophages from healthy donors were infected with HIV (89.6 strain). Following infection, cultures were treated with semilog concentrations of TCN-P or AZT. Appropriate controls (infected, non-infected, non-treated) were used. Levels of viral infection were measured by PCR for HIV *gag* and antigen capture ELISA for HIV p24 at day 1, 2, 4, and 7 post-infection. The results suggest that TCN-P is more potent than AZT in reducing viral infection at later time points. This observation is consistent with our current hypothesis that TCN-P may interfere with viral assembly.

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Inhibition of human immunodeficiency virus type-1 in macrophages by calcium trinatrium diethylene-triaminepentaacetic acid

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Calcium trinatrium diethylenetriaminepentaacetic acid (CaDTPA) is a potent inhibitor of human cytomegalovirus replication *in vitro* and exerts immunomodulatory activities such as suppression of adhesion molecules on different types of cultured human cells. We observed effects of CaDTPA on human immunodeficiency virus type-1 (HIV-1) replication in cultured monocyte-derived macrophages (MDMs). MDMs were infected with HIV Ba-L and treated with the drug either immediately (acute infection) or after 14 days (chronic infection). Inhibition of HIV-1 replication was assessed by quantification of HIV-1 p24 antigene in culture supernatant using an enzyme immunoassay. In acutely infected cells after 14 days of treatment 50% of HIV-1 replication was inhibited by 0.7 µM CaDTPA while 50% of MDDs viability was reduced at a concentration of 150 µM (therapeutic index 200). HIV-1 replication and virus production was inhibited completely at a concentration of 10 µM CaDTPA and was not detected after additional 14 days incubation in medium without the drug. The drug had also significant anti-HIV-1 activity in chronically infected MDMs at a concentration ranging from 2 to 20 µM CaDTPA.. In addition to antiviral activity CaDTPA inhibited synthesis of mRNA for intercellular adhesion molecule (ICAM-1) in HIV-1 infected MDDs or MDDs treated with interleukin-4 as demonstrated by reverse transkriptase-polymerase chain reaction. The results showed that CaDTPA is a potent inhibitor of HIV-1 replication in macrophages and is effective in suppression of HIV-1 induced upregulation of ICAM-1 mRNA in MDDs.

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New Types of Anti-HIV Agents Identified by the NCI Drug Discovery Program

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Through a combination of cell-based screening and rational drug design efforts to target selected viral macromolecules, a wide variety of new classes of anti-HIV agents have been identified. These include inhibitors of HIV-1 transcription, inhibitors of virus fusion that act by affecting chemokine co-receptors on cell surfaces, as well as inhibitors of the HIV-1 nucleocapsid protein. Temacrazine was developed as a nanomolar inhibitor of HIV-1 transcription that selectively inhibits synthesis of HIV-1 RNA without interference with transcription of cellular genes or events associated with the Tat and Rev regulatory proteins. This compound inhibits acute, latent and chronic HIV-1 infections and demonstrates in vivo anti-HIV-1 activity. A distamycin analog, designated as NSC 651016, prevents HIV from fusing with host cells by targeting chemokine co-receptors. NSC 651016 blocked chemokine binding to CCR5, CCR3 and CXCR4, but not to CXCR2 or CCR2b, and the compound inhibited HIV-1 replication in an in vivo model. A variety of chemotypes have been identified as inhibitors of the nucleocapsid protein zinc finger motifs. Such compounds demonstrate virucidal activity and prevent the formation of infectious virus from infected cells. Three compounds have been found to selectively disrupt the nucleocapsid protein zinc fingers without affecting cellular zinc finger proteins. No drug-resistant isolates have been detected to the nucleocapsid protein zinc finger inhibitors, and two such compounds are currently in clinical trials. These examples illustrate a few of the new types of anti-HIV agents identified by the National Cancer Institute.

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Novel Ribonucleotide Reductase (RR) Inhibitors, Didox and Trimidox, Produce Antiretroviral Effects in the Murine Immunodeficiency (MAIDS) and in the HIV-infected HuPBMC SCID Models. H. Elford[1], B. van't Riet[1], C. Mayhew[2], O. Oakley[2], J. Piper[2], V. Gallicchio[2], P. Black[3], S. Kunder[3], G. Goldberg[3], D. Broud[3], B. Hall[3], M. Bacho[3], S. Papermaster[3], and M. Ussery[3]. [1]Molecules For Health, Inc., Richmond, VA; [2]University of Kentucky, Lexington, KY; [3]U.S.F.D.A., Rockville, MD, USA.

Inhibition of ribonucleotide reductase (RR) and thereby reducing the pools of deoxynucleotides necessary for the synthesis of proviral DNA in HIV infection has gained recognition as a potential approach to HIV infection. This premise has been supported by a number of reports from in vitro studies, retroviral infection animal models as well as clinical studies that HIV infection is strongly suppressed especially in combination with didanosine (DDI). Data will be present comparing both short-term and long-term treatment of the RR inhibitors alone and in combination with DDI in the murine immunodeficiency model of AIDS (MAIDS). Infected mice were treated five times per week (M-F) or daily, singly or in combination with DDI. Didox or Trimidox treatment alone produced marked increase in survival and a potent suppression of viremia. Other parameters of infection such as hypergammaglobulinemia and tumor development were strongly suppressed. The antiviral effect was enhanced with DDI but toxicity also was more pronounced. DDI alone in this model had only marginal activity. A study was conducted to determine if the antiviral effects observed with the murine retroviruses can also be obtained with HIV. In the HIV-infected HuPBMC/SCID mouse model, female mice were reconstituted with human peripheral blood monocytes (PBMC) 2 weeks before infection. Mice were treated daily i.p. with Trimidox for a total of 7 days starting 1 day prior to HIV infection. In a dose-dependent fashion, TX decreased average viral titer in peritoneal cells, lymph nodes, spleen and peritoneal cells. Trimidox reduced titers of infectious virus (as measured by quantitative coculture) by 10 fold from lymph nodes and peritoneal cells. The antiviral effect as monitored by viral RNA copies (measured by NASBA) was more pronounced. Viral RNA copies were reduced by nearly 3 logs in lymph nodes and peritoneal cells and by 1 log in plasma. These data support the potential of these compounds for AIDS therapy.

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Immunomodulating and Antiviral effects of CNBA-Na on Isolated Human PBMC and HIV-Infected HuPBMC-SCID Mice. D. Kinchington, W. O. Ayuko, D. Devine, N. Gilbert, O. Wood, S. Kunder, D. Broud, S. Papermaster, M. Bacho, B. Hall, C. Nielson and M. Ussery. St Bartholomew's and the Royal London School of Medicine and Dentistry, 51-53 Bartholomew Place, London EC1A 7BE and Antiviral Research Laboratory, FDA, 5600 Fishers Lane, Rockville, MD 20857, USA.

Studies with PBMC isolated from healthy donors and HIV-infected individuals show that anti-CD3 induced proliferation can be enhanced when the sodium salt of 2-chloro- 5-nitrobenzoic acid (CNBA-Na) is added to the culture medium. The mechanism of action of this compound is thought to be via an IL-2 dependent mechanism as a transitory rise in IL-2 release between 12- and 24 hours is observed. CNBA-Na was also examined in HIV-1 infected HuPBMC-SCID mice. Dosing (50- 200 mg/kg) was begun 3 days before infection and continued 6 days after infection. Necropsy was carried out on day 7. Infectious virus measured by quantitative coculture was inhibited by about 80% in lymph nodes. There was less inhibition or modest viral activation in spleen, blood and peritoneal wash cells, although this level of virus replication should be controllable by concomitant antiviral therapy. CNBA-Na was not toxic to CD4 or CD8 cells in uninfected animals. CNBA-Na also prevented the viral - induced reduction in CD4/CD8 ratio in all sites. These studies in human PBMC and the HuPBMC-SCID- model indicate that CNBA-Na appears to have a positive immunological effect which is more evident than a direct antiviral effect. This data will highlight the current interest in combining immunomodulating and antiretroviral agents in treating HIV infections.

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Quantitative Structure-Activity Relationship Studies of Potential Anti-HIV Drugs

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Quantitative structure-activity relationships (QSAR) were developed for three series of compounds (nucleoside analogs, integrase inhibitors, and non-peptide protease inhibitors) with anti-HIV activity. The series were independently investigated to determine the correlation of structure and activity using molecular similarity analysis and structure-activity maps. A multiple-formula approach was used to perform quantitative molecular similarity analysis (QMSA) and QSAR study. Molecular descriptors such as molecular topological number (NAB), maximum common substructure (MaCS), minimum common superstructure (MiCS), and molecular similarity index (MSI) were used in our structure-activity relationship study. Structure-activity maps and QMSA were used to determine the site and type of modification for improved activity and reduced toxicity of new compounds.

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ANTI-HIV ACTIVITY OF POLYOXOMETALATES

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A series of polyoxometalates have been synthesized and evaluated for their inhibitory effects on HIV-1(III_B) and HIV-2(ROD) replication in MT-4 cells. All compounds showed activity against HIV-1 and HIV-2 but the anti-HIV potency of the heteropolytungstates varied considerably depending on their chemical structure. The activity of single, double and triple Keggin-type of compounds against HIV-1(III_B) was comparable (EC_{50}: 0.3-0.5 μg/ml), whereas HIV-2(ROD) appeared to become less sensitive with the increasing number of Keggin structures per compound. The same trend was observed for single and double Dawson structures. Some compounds were examined for their inhibitory effect against the replication of HIV-1(RF) and SIV(MAC_{251}) in MT-4 cells. Their anti-HIV-1(RF) or - SIV(MAC_{251}) effects were comparable to those obtained with HIV-1(III_B) or HIV-2(ROD), respectively. The polyoxometalates represent a class of polyanionic compounds, which block the binding of HIV particles to the $CD4^+$ cells. They interfere with the binding of OKT4/Leu-3a mAb to the CD4 receptor of MT-4 cells, and inhibit the binding of anti-gp120 mAb to persistently HIV-1 infected HUT-78 cells, as shown for one of the double Keggin structure compounds. However, this double Keggin structure compound did not inhibit the binding of a specific CXCR4 mAb to SUP-T1 cells (as measured by flow cytometry), suggesting that it does not interact with CXCR4, the co-receptor for HIV.

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IN VITRO ANTI-HIV ACTIVITY OF EXTRACT FROM STEVIA REBAUDIANA

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Anti-HIV activity of Stevia extract was examined by MTT assay in vitro. Stalks and leaves of Stevia baudiana were extracted with hot water and concentrated. This solution is referred to Stevia original solution. It (100g) contains 84.2g of water, 7.3g of carbohydrate, 4.5g of ashes, 3.6g of protein and 0.4g of lipid. Anti-HIV activity was evaluated by MTT cytotoxicity assay using MT-4 cells. EC_{50} of Stevia extract was x30,000 dilution of the original solution, while CC_{50} was below x1000 dilution. The anti-HIV activity was found in fractions eluted with 30% and 60% ethanol on YMC-ODS reverse phase column chromatography. This fraction did not inhibit TNF-induced HIV replication in persistently HIV-infected OM10 cells. Preliminary experiments showed that this inhibitory effect is due to inhibition of viral attachment to MT-4 cells. Purification and characterization of the inhibitory agents are under investigation.

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Anti-HIV-1 Activity of a Commonly used Chinese Traditional Herb DS in vitro and in vivo. Hong-Shan Chen, Xing-Quan Zhang, Li Teng, Yao-Zeng Lu, Xiao-Xian Wu, Xiang-Hong Chen, Xu-Guang Yan, Institute of Medicinal Biotechnology, Institute of Experimental Animals, Chinese Academy of Medical Sciences, Beijing, 100050, China.

DS is a kind of Chinese traditional herb, which has been commonly used for treatment of cardiovascular and liver diseases. The crude extract of its roots was found to inhibit HIV- 1 P_{24} expression both in human T-lymphocyte and peripheral blood monocyte cultures. It showed inhibitory activities both on AZT sensitive and resistant virus strains and synergistic with AZT on these viruses in PBMC cultures. The crude extract when given by oral significantly reduced the spleenomegalopathy and decreased the white blood count of leukemia in Rauscher murine leukemia virus infected mice. It also inhibited the viremia in Simian immunodificiency virus (SIV) infected monkeys. The crude extract was shown as an interferon inducer when given by oral to mice. Its toxicity is rather low in animals, mice tolerated 40 g/kg po or 2 g/kg iv once and 2g/kg po bid for 20 days; rhesus monkeys tolerated 300mg/kg qd for 56 days; all showed no toxic effect. DS is widely distributed in China, to develop it as an anti-HIV-1 drug for treatment of AIDS is promising,

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Role of cytolytic T-cells in controlling disease progression in a long-term nonprogressor (LTNP)

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Objective: To study the importance of cytolytic T-cells (CTLs) recognizing highly conserved epitopes in controlling disease progression.

Methods: This study based on a described CTL-clone, which was established from a LTNP (Harrer et al., 1996) and which recognizes a 9 amino acid (aa) HLA-B14 restricted epitope. This epitope, which is located in p24 CA, confirmed to be highly conserved among all sequenced HIV-isolates. Each position of this epitope has been substituted by site- directed mutagenesis (i) conservatively and (ii) non-conservatively. After insertion of the altered sequences into a pNL4-3 provirus backbone, 18 recombinants were each transfected into different cell lines and analyzed with respect to their replicative capacity. In parallel the recognition of the epitope variants by the p24 CA specific LTNP derived CTL-clone was determined in a ^{51}Cr-release assay. Synthetic peptides representing the optimal nonameric epitope were used for labelling of autologous B-cells.

Results: The majority of the generated mutants showed only minor defects in virion morphogenesis when tested on COS-7 cells. By contrast, only 4 out of 9 (4/9) conservative aa-substitutions yielded replication-competent virus mutants when tested on different CD4+ cell lines. For comparison, 9/9 non-conservative aa-substitutions resulted in a replication-defective phenotype. Notably, all peptides representing the sequence of the infectious virus variants were capable to mediate specific lysis of autologous B-cells in a ^{51}Cr-release assay .

Conclusion: Sequence variations introduced in a conserved epitope, which is recognized by CTLs of a LTNP, are sensitve with respect to viral infectivity. Those variants, that are still capabe to replicate, are recognized by LTNP's CTL response. This suggests, that the chances of the virus to escape the immune control in this epitope via seqeunce variations are limited.

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Novel Pharmaceutical Formulations With Virucidal Activity.

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In recent years there has been some interest in the possibility of using microbicidal compounds to prevent sexually transmitted diseases. Vaginal spermicides, particularly nonoxynol-9, have been shown to kill sexually transmissible viruses and bacteria *in vitro* and *in vivo*. However, because of the harmful effects of frequent application of nonoxynol-9 to mucosal membranes there is a need for other less toxic vaginal microbicides. The virucidal effect of long-chain unsaturated fatty acids and medium-chain fatty acids and their 1-monoglycerides is well known. Novel pharmaceutical formulations have now been developed which contain 1-monoglyceride of capric acid (monocaprin) as the active ingredient. Formulations with 5 mM monocaprin cause at least a 100,000 fold reduction in the infectivity titer of herpes simplex virus type 1 when incubated with the virus in culture medium for 1 minute. Similarly, formulations with 20 mM monocaprin cause almost a 100,000 fold reduction in the titer of human immunodeficiency virus type 1 (HIV-1) in 1 minute. The formulations are slightly less active against virus mixed into human semen. Formulations with 10 mM monocaprin reduce the number of leukocytes in semen by more than 10,000 fold in 1 minute and also cause a marked reduction in the motility and viability of spermatozoa. They were not harmful to the vaginal mucosa of mice when applied daily for 10 days. The potential use of the formulations for prevention of HIV-1 infection and other sexually transmitted infections will be discussed.

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Development of Genotypic and Phenotypic Resistance to MKC-442, a Potent and Selective Inhibitor of HIV-1 Replication. J. Harris, K. Borroto-Esoda, E. Hill, C. Moxham, F. Rousseau, and B. McCreedy. Triangle Pharmaceuticals, Durham, NC, USA

MKC-442 (6-benzyl-1-[ethoxymethyl]-5-isopropyl-uracil) is a nucleoside analog that functions as a non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1 replication. In HIV-infected MT-2 cells the EC_{50} for MKC-442 is approximately 14 nM. Phenotypically resistant virus was generated by serial passage of HIV-1 (LAI strain) in MT-2 cells in increasing concentrations of MKC-442 up to 1152 nM. Sequence analysis of the resistant virus revealed a Y181C mutation in the RT gene. In a Phase I/II trial in HIV-infected patients, MKC-442 showed significant antiviral efficacy with a median reduction in viral load of –1.41 $\log_{10}$ from baseline when administered as 750 mg bid. Viral loads began to return towards baseline in all patients after one month (median –0.47 $\log_{10}$ reduction). In contrast to the Y181C mutation selected in vitro, sequence analysis of plasma virus from these patients showed the presence of a K103N mutation emerging at the time of viral load rebound. Viruses obtained by co-culture of PBMC's from two patients at two different doses of MKC-442 (100 mg and 250 mg bid) showed that the viruses had also acquired the K103N mutation. However, the K103N mutant viruses had different levels of phenotypic sensitivity to MKC-442 depending upon the genotype of the virus present in the patient at baseline. One patient, whose HIV was wild type at baseline, developed a K103N mutation with a corresponding 70-fold increase in the EC_{50} for MKC-442. A second patient, whose baseline virus had the M184V mutation, also developed a K103N mutation, but the K103N/M184V mutant virus showed only a moderate increase in EC_{50} for MKC-442. Several patients who received the optimal dose of MKC-442 (750 mg bid) possessed virus with an M184V mutation at baseline and later acquired the K103N mutation. Co-culture of the PBMC's from these patients is being performed to determine if the K103N/M184V mutant virus grows out and if the virus shows a decreased level of phenotypic resistance to MKC-442.

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Antiviral Susceptibilities of HIV-1 Reverse Transcriptase Recombinant Viruses Derived from AIDS Patients after Extended Adefovir Dipivoxil Therapy. M.D. Miller, K.E. Anton, A.S. Mulato, P.D. Lamy and J.M. Cherrington. Gilead Sciences, Foster City, CA 94404.

Adefovir dipivoxil (Preveon™), an oral prodrug of adefovir (PMEA), is currently in phase III trials for treatment of HIV-1 infections. In a phase II trial, 29 patients completed from 6 to 12 months of extended therapy during which concomitant medications were allowed. Genotypic analyses of plasma HIV from these patients demonstrated that 8 patients developed mutations in RT which were possibly associated with Preveon treatment. Despite these mutations, the patients showed sustained viral load suppression during treatment. Pre- and post-treatment HIV RT recombinants were generated from these patients samples to determine antiviral drug susceptibilities. Recombinant HIV from 2 patients with K70E and 2 patients with T69D mutations demonstrated a 2–3-fold and less than 2-fold decrease in adefovir susceptibility, respectively. As previously observed during long-term monotherapy with either ddI or d4T, 4 patients in this study who were not taking AZT developed typical AZT-resistance mutations. Recombinants from these patients showed decreases in adefovir susceptibility ranging from 2–6-fold. Recombinants were also analyzed from 4 additional patients who developed the M184V mutation, due to concomitant 3TC use, in a pre-existing background of AZT resistance mutations. In these cases, addition of the M184V mutation notably increased the *in vitro* susceptibility of these high-level 3TC/AZT resistant viruses to near wild-type values for adefovir. The clinical significance of this susceptibility reversion is under investigation. Together, these data from HIV-1 recombinants are consistent with the durable antiviral effect observed in patients treated with Preveon, including those who developed mutations in RT.

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HIV-1 Reverse Transcriptase (RT) Expressing a K70E Mutation Exhibits a Decrease in Both Specific Activity and Processivity. P.D. Lamy, M.D. Miller, M.D. Fuller, A.S. Mulato, N.A. Margot, T. Cihlar, and J.M. Cherrington. Gilead Sciences, Foster City, CA 94404.

Adefovir dipivoxil (Preveon™), an oral prodrug of adefovir (PMEA), is currently in phase III clinical testing for the treatment of HIV-1 infection. Previous *in vitro* experiments have shown that viral variants expressing either a K65R or a K70E mutation in HIV-1 RT showed reduced sensitivity to PMEA and that the K70E mutant also showed impaired replication capacity. In a genotypic analysis of a phase I/II clinical trial of Preveon, the K70E mutation developed in 2 patients during extended monotherapy. To further investigate the potential molecular mechanisms involved in the resistance to PMEA, we have cloned, expressed and purified HIV-1 RT enzymes carrying the K65R, K70E and, for comparison, the M184V mutation. The Km values of dNTPs for these mutant enzymes were not significantly altered from wild-type RT. The Ki values for the K65R mutant were increased 2–10-fold against a variety of inhibitors, while the M184V mutant increased 8-fold for only 3TCTP. The Ki values for the K70E mutant were specifically increased for PMEApp and 3TCTP by only 2–3-fold. These results are in agreement with antiviral drug susceptibility assay results. The 3 recombinant enzymes were also evaluated for their specific activity and processivity. All mutants were reduced in their specific activity with respect to wild-type RT. In single cycle processivity studies, the M184V mutant was notably impaired. The K70E mutant was only slightly impaired, while the K65R mutant was slightly more processive than wild-type. These enzymatic results with recombinant K70E RT agree with the reduced *in vitro* replication capacity of the K70E RT HIV-1 mutant and further demonstrate that this mutation confers minor resistance to PMEA and 3TC.

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Resistance to Loviride (R089439) and Other NNRTIs : Characterization of the In Vitro Emerging HIV-1 Variants under Continuous Pressure of the Inhibitors. M.-P. de Béthune[1], K. Andries[2], H. Azijn[1], J. Van Peel[1] and R. Pauwels[1]. [1]Tibotec NV, Edegem, Belgium and [2]Janssen Research Foundation, Beerse, Belgium.

The rapid in vivo emergence of resistant HIV-1 upon administration of NNRTIs has prompted us to analyse this phenomenon in vitro. We have designed an assay in which we monitor viral breakthrough in the presence of the different inhibitors. More precisely, MT4 cells are infected with HIV-1 at a very high MOI (10-100 $CCID_{50}$/cell) in the presence of varying concentrations of the tested compounds. The total virus input represents ± 10^9 viral RNA copies/culture flask. The use of a very high MOI allows for the emergence of a large variety of variants present in HIV-1 populations. Cultures are monitored every 3 to 4 days and emerging viruses are harvested. This methodology has been applied to the laboratory LAI strain and to recombinant HIV strains in which the RT gene from patient's HIV has been introduced in a HXB2 background. Using this experimental scheme, we were able to isolate HIV-1 populations resistant to Loviride, Nevirapine, DMP266 or Delavirdine after relatively short culture times (1 to 3 cell subcultivations), even at high concentrations of the inhibitors (5 or 10µM). Genotyping of the resistant viruses shows they all harbour 1 or more NNRTI resistance associated mutation(s). Phenotypic characterization of those viruses shows high levels of resistance and some cross-resistance among the tested inhibitors. Results will show the different mutations observed and the phenotypic profiles of the emerging viruses. Those are representative of the minor populations present in the original quasi-species. The time required for their emergence is a measure for both the frequency at which they are initially present and the potency of the selection compound in inhibiting these variants.

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Genotypic Analysis and Phenotypic Susceptibility of HIV-1 Isolates from Patients Treated with Stavudine (d4T)-Didanosine (ddI) Combination. S. AUGER, V. FERRE-AUBINEAU, S. MONPEHO, V. RELIQUET, S. BILLAUDEL, F. RAFFI. Virology Department, Pharmacy School, and Infectious Diseases Department, University Hospital, Nantes, France.

We determined the phenotypic susceptibility and presence or emergence of mutations associated with d4T and/or ddI resistance of HIV-1 isolates from patients (pts) enrolled in the QUINTET d4T-ddI combination trial. QUINTET is a 6 months (M) trial of safety and antiviral activity of d4T-ddI combination in 65 pts with previous antiretroviral therapy with zidovudine ± zalcitabine, >100 CD4 cells/mm3, and >10,000 HIV-1 RNA copies/ml. Baseline and M6 paired isolates were assayed for d4T and ddI susceptibilities by the ANRS consensus, PBMC, reverse transcriptase (RT), based assay, and for genotypic analysis using selective PCR to detect mutations at codons 74, 75 and 184 of the RT genomic region. Mean plasma HIV RNA decrease was 1.1 log_{10} copies/ml at week 4 and 0.85 log_{10} copies/ml after 6 M of d4T-ddI combination. Among the 21 pts tested for the resistance study, IC50's for d4T and ddI have been determined for 10 paired isolates:

Patient strains	ddI IC50 (micromolar) mean; median [range]	d4T IC50 (micromolar) mean; median [range]
Baseline	1.75; 1.5 [0.83-2.67]	0.09; 0.08 [0.002-0.17]
Month 6	1.86; 2.1 [0.84-2.52]	0.162; 0.12 [0.05-0.39]

Among the 13/21 pts with no mutation at baseline, a mutation was detected at M6 in 4 (codon 74 = 1, 75 = 1, 184 = 2). The isolate with acquisition of mutation V75T had a significant increase of d4T IC50 (x 25). Plasma HIV RNA change at M6 was not significantly different in pts with (n=8) or without (n=13) RT mutation at baseline (-0.8±0.4 log_{10} vs -1.±0.5 log_{10}). In pts without RT mutation at baseline, HIV RNA change did not correlate with emergence of mutation at M6 (-0.9±0.7 vs -1.1±0.6 log_{10}). Conclusion: No overall change in d4T or ddI susceptibilities was observed in isolates derived from d4T/ddI treated pts. Emergence of d4T/ddI associated mutations occurred at a low rate and was associated with decreased antiviral activity in only 1 case. This could explain the more sustained effect of d4T-ddI combination as compared to other nucleoside analogue combinations.

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Resistance Profile, Selectivity and Cellular Uptake Studies of BCH-10618, BCH-10619 and BCH-10652

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The BCH compounds 10618, 10619 and 10652 are potent and selective inhibitors of HIV-1 replication *in vitro*. Tissue culture selection studies with BCH-10619 have demonstrated that approximately twelve passages are needed to select for resistant viruses attaining about 5-10 fold resistance to the nucleosides. BCH-10619 resistant virus maintained its sensitivity to BCH-10618, however, 3TC®, β-L-ddC and β-L-5FddC were inactive against the resistant virus. A single mutation conferring resistance to BCH-10619 was identified which was verified by site-directed matagenesis studies. BCH-10618 and BCH-10652 did not select for a resistance mutation under similar conditions to BCH-10619. In a primer extension assay, the triphosphates of BCH-10618, BCH-10619 and BCH-10652 were found to be potent inhibitors of wild type reverse transcriptase (RT) enzyme, with IC_{50} values similar to ddCTP. The IC_{50} values increase about 2-fold against RT containing the M184V mutation. BCH-10618TP, BCH-10619TP and BCH-10652TP are weak inhibitors of mammalian polymerase B. Cellular uptake of [^{3}H] -BCH-10618 in CEM cells indicated the presence of a transient metabolite in addition to the the diphosphate and triphosphate forms. A different metabolite was observed for [3H] -BCH-10619 which accumulated over the course of 48 h. These results provide the basis for the selection of candidates for further development from this series of novel nucleoside analogues.

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Effect of Drug Resistance Engendering Mutations on Viral Fitness Stup, T.L., Pallansch, L.A. and Buckheit, R.W., Jr. Southern Research Institute, Frederick, MD, USA

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 and protease 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 and allow the immune system to more effectively deal with the virus. It has been previously reported that RT with the Gly190Glu amino acid change exhibited a significant decrease in RNA-dependent DNA polymerase activity compared to wild-type RT. The handicapped enzyme was unable to perform at full capacity (about 4%). We have introduced point mutations that confer drug resistance to nucleoside and nonnucleoside RT inhibitors into the RT of pNL4-3 by site-directed mutagenesis. The relative replication potential of HIV strains with each of these point mutations was determined and will be presented.

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Evaluation of Anti-HIV Activity Against Low Passage Clinical Strains of HIV-1 Osterling, M.C., Halliday, S.M. and Buckheit, R.W., Jr. Southern Research Institute, Frederick, MD, USA

We have evaluated the activity of anti-HIV agents in a variety of clinically relevant assay systems in order to define the most sensitive and appropriate model for use *in vitro*. Each of these assays uses fresh human cells infected with low passage clinical strains of virus. The most commonly employed assay employs PHA-stimulated PBMCs from normal human donors. In these assays, the T-cell mitogen induces significant levels of cell proliferation and virus production. A variation on this theme has involved the use of IL-2-stimulated PBMCs. Cells activated by antigen exposure in the host are induced to continue their proliferation by the introduction of exogenous IL2. Monocyte-macrophage cultures have also been employed to evaluate the activity of agents. These adherent cells do not proliferate in cell culture and their use is limited to the evaluation of monocyte-macrophage-tropic strains. Evaluations have also been performed in dendritic cells isolated from fetal liver. Finally, we have evaluated the use of human spleen mononuclear cell cultures, which allow the long term growth of a large variety of clinical virus strains in the absence of mitogenic stimulation. We will present comparative anti-HIV data obtained with each of these assay systems using a variety of anti-HIV agents.

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Potentiation of HIV-1 replication and failure of anti-HIV-1 activity of antiretroviral agents in cisplatin resistant cell line.

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Different cellular resistance mechanisms account for decreased activity of 2',3'-dideoxynucleoside analogues against human immunodeficiency virus type-1 (HIV-1). To study the influence of cisplatin-induced cellular resistance on replication of human immunodeficiency virus type-1 and activity of 2',3'-dideoxynucleoside analogues against HIV-1 we established cisplatin-resistant human lymphoid cell line (C8166), growing in medium with 2 µg/ml of cisplatin (CDDP). The flow cytometry did not revealed changed expression of glutathion-S-transferase (GST), c-myc and p53 expression in resistant (designated as C8166/CDDP$^{2.0}$) cells when compared with C8166 cells. Bcl-2 and mdr-1 expressions were increased in C8166/CDDP$^{2.0}$ cells while Fas expression was downregulated. The dramatic increase of HIV-1 replication was observed in C8166/CDDP$^{2.0}$. Infectious virus titers of HIV-1 laboratory strains were up to 1000-fold greater in C8166/CDDP$^{2.0}$ cells comparing with parental cell line. As measured by HIV-1 p24 production (ELISA) were the antiviral activities of nucleoside analogs: 3'-azido-2',3'-dideoxythimidine (AZT), 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI) and 3'-thio-2',3'-dideoxycytidine (3TC) 10- to 50-fold decreased, while the efficacy of acyclic nucleoside phosphonates including 9-(2-phosphonylmethoxyethyl) adenine (PMEA) and 9-(2-phosphonylmethoxyprolyl) diaminopurine (PMPDAP) was only 2- to 4-fold lower in the resistant than in parental C8166 cells. The results showed that cellular phenotype specific to cisplatin resistance is associated with great increase of HIV-1 replication and decreased activity of different antiretroviral drugs.

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TREATMENT OF HIV-INFECTED CELL CULTURES WITH HIGH CONCENTRATIONS OF ANTI-HIV DRUGS FOR SEVERAL DAYS DOES NOT PREVENT RESUMPTION OF VIRUS PRODUCTION UPON REMOVAL OF THE ANTIVIRALS

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The effects of HIV-1 RT and protease inhibitors (RTIs and PIs) are known to be reversible at the enzyme level; but data at the cellular level are scarce. We tried to establish whether MT-4 cells that were infected with HIV-1 (III_B) at a high multiplicity of infection (m.o.i. = 1), and subsequently treated with high concentrations of antivirals, would be able to resume virus production once the antivirals were washed away. The HIV inhibitors studied were zidovudine, lamivudine, nevirapine, delavirdine, loviride and the protease inhibitors indinavir and ritonavir, at 50 and 500 times their 50 % inhibitory concentration (IC_{50}). Compounds were added immediately after virus adsorption and removed after an incubation period of 0 (wash control), 24, 48 or 72 hours. Virus breakthrough was monitored by microscopical examination of cytopathicity and determination of p24 levels in the supernatant. None of the antivirals studied was able to prevent viral growth upon removal of the compound. The NNRTIs nevirapine, delavirdine and loviride, at 500 times their IC_{50} and lamivudine at 220 µM, were able to prevent viral breakthrough for approximately 4 days. Whereas the RTI zidovudine and the PIs indinavir and ritonavir, under the same conditions were not able to delay viral breakthrough. Our findings may suggest that drug holidays may result in the breakthrough of virus that had been suppressed for several days by adequate drug levels.

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***CYCLO*SAL-NUCLEOTIDES OF 2',3'-DIDEOXY-ADENOSINE DERIVATIVES AS EFFICIENT NUCLEOTIDE DELIVERY SYSTEMS**

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Different *cyclo*Sal-pro-nucleotides of antivirally active 2',3'-dideoxyadenosine derivatives (ddA, d4A, F-beta- (up-) and F-alpha- (down-) ddA) were prepared, chemically hydrolyzed in phosphate buffer at pH 7.3 and in RPMI culture medium containing 10% heat-inactivated fetal calf serum as well as evaluated for their biological activity against HIV-1 and HIV-2. It was shown in chemical hydrolysis studies that these compounds released the monophosphates (ddAMP, d4AMP, 2'-F-*ara*- and 2'-F-*ribo*-ddAMP) as the sole nucleoside containing product. Consequently, these phosphotriesters potentially can also act as ***nucleotide prodrugs*** in biological systems and thus, circumventing the deamination of the parent nucleoside to the corresponding inosine derivatives by adenosine deaminase (ADA-bypass). The kinetics of the chemical hydrolysis showed a clear relation of the hydrolysis rate and the electronic properties of the aryl-substituent. The mechanism of the chemical hydroysis of the *cyclo*Sal-derivatives involves a controlled tandem-reaction. Studies concerning the resistance against deamination to give the inosine derivatives by ADA showed marked enhanced stability of the pro-nucleotides. Additionally, these new compounds were tested *in-vitro* against HIV-1 and HIV-2 in CEM/0 cells compared to the corresponding exhibited significant antiviral activity in evaluation.

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Incorporation of Amphotericin B into Stealth Liposomes or Colloidal Dispersions Diminishes its *In Vitro* Anti-HIV Activity

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We examined whether the anti-human immunodeficiency virus type 1 (HIV-1) activity of the polyene antibiotic Amphotericin B (AMB) is retained following incorporation into sterically stabilized "Stealth" liposomes (L-AMB) with prolonged circulation *in vivo*, or cholesteryl sulfate colloidal dispersions (CD-AMB). The effects of the different preparations on acute infection of H9 cells with HIV-1_{IIIB} and spreading of the virus from chronically infected H9/IIIB cells to SupT cells were evaluated. Infection was monitored by p24 levels in culture supernatants. The presence of L-AMB (0.3 to 20 µg/ml) during initial infection did not affect p24 production in H9 cells, while free AMB (3 to 20 µg/ml) reduced p24 levels by 70-80% after 7, 10 and 14 days post-infection. Continuous treatment of H9/IIIB and SupT cells with free AMB for 7 days resulted in complete inhibition of p24 production in the concentration range 3-20 µg/ml, and 90% inhibition with 1 µg AMB/ml. Under the same conditions L-AMB (1 to 20 µg/ml) did not affect p24 production. In contrast, the presence of CD-AMB during spreading of infection reduced both p24 production and the cytopathic effect (syncytium formation) in a dose-dependent manner. After 2 days in culture, CD-AMB inhibited virus production by 100% at 10-20 µg/ml and by 90% at 3-5 µg/ml. After 4 days, however, the level of inhibition was reduced to 40-50%. Because CD-AMB is considerably less cytotoxic than AMB, its ability to inhibit HIV infection *in vivo* needs to be evaluated further.

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Comparison of the Plasma Pharmacokinetics of AZT and AZT Phosphoramidates

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The *in vitro* anti-HIV activity and high water solubility of AZT phosphoramidates has suggested that they would be good candidates for preclinical pharmacokinetic studies.[1,2] Conse-quently, we have examined the pharmacokinetic parameters in rats of the 3'-azido-3'-deoxythymidine-5'-methoxyphenyl-alaninyl phosphoramidate (APP), and compared them to those found for AZT.

The phosphoramidate level in serum after i.v. administration of 19 µmol/Kg of APP was 1.6 µM at 15 min and decreased with a half-life of 121.3 ± 46.8 min. In contrast, when AZT was administered, its half-life was 24.8 ±10.0 min, and the limit of quantitation was reached within 4 hours. The half-life of AZT was similar to that in previous reports. The half-life of APP was significantly longer than that of AZT (unpaired t-test, $p<0.05$), as has been reported for other AZT prodrugs.

The total body clearance (CL) of AZT was 14.99 ± 5.26 ml/min. Surprisingly, the CL of the phosphoramidate APP was significantly increased (35.3± 10.1 ml/min) over AZT (unpaired t-test, $p<0.05$). However, the volume of distribution for APP was 16.7 ± 8.0 L vs. 1.70 ± 1.31 L for AZT (statistically significant at $p < 0.05$). This indicates that APP has a 9-fold greater ability to distribute outside the plasma than does AZT. The large volume of distribution of APP outweighs its higher CL, thus producing a significantly longer half-life than AZT and greater access to tissue space than AZT.

(1) Wagner, C. R. et. al., Bioorg. Med. Chem. Letts, 5, 1819 (1995); (2) McIntee, E. J., et. al., J. Med. Chem. 40, 3323 (1997)

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Measurement of Intracellular Pools of dATP by High-Performance Liquid Chromatography Following Derivatization to Generate a Fluorescent Chromophore

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An HPLC-fluorescence assay for quantification of dATP within cultured human peripheral blood mononuclear cells (PBMC) is described. Derivatization for fluorescence detection has been investigated as a means of enhancing sensitivity for the paired-ion HPLC analysis of dATP, a naturally occurring deoxyribonucleotide. The reaction of chloroacetaldehyde with the adenine base of dATP has been employed to form fluorescent 1, N^6-etheno-dATP with a yield of >95%. The derivative gives an analytically useful fluorescence emission at 403 nm after excitation at 275 nm. Conditions for derivatization, fluorescence detection, and chromatography have been optimized. The assay is linear over a wide range, from 25 to 2500 nM, with a low detection limit of 50 nM. The intra-day and inter-day precision and accuracy for determinations of standard and quality control samples showed a coefficient of variation of <15%. The bench stability and process stability of the samples were found to be >91% over 2 weeks. This technique is suitable for high sensitivity quantification of dATP pools in samples from preclinical and clinical pharmacologic studies. Moreover, this method should be particularly useful for measuring nucleotide pool reductions in cells treated with ribonucleotide reductase inhibitors, such as hydroxyurea.

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The Intracellular Pharmacology of ß-L-ddA is Responsible for the Lack of Potent Anti-HIV Activity. L. Placidi1,[1] C. Perigaud,[2] E. Cretton-Scott,[1] G. Gosselin,[2] C. Pierra,[2] R.F. Schinazi,[3] J.L. Imbach,[2] and J.P. Sommadossi.[1] Department of Pharmacology, University of Alabama at Birmingham, USA[1], Laboratoire de Chimie Bio-Organique, Universite de Montpellier II, France[2], Emory University School of Medicine/VA Medical Center, Decatur, GA.[3]

Background to study: The unnatural ß-L enantiomer of 2',3'-dideoxyadenosine (ß-L-ddA) was demonstrated to lack potent activity against human immunodeficiency virus (HIV) replication *in vitro* in human peripheral blood mononuclear cells (PBMC) with an EC_{50} > 100 µM. This is despite the fact that L-ddA is not a substrate for adenosine deaminase and is a good substrate for deoxycytidine kinase.

Objectives: To investigate the intracellular metabolism of this nucleoside analogue in human PBMC, and primary cultured human hepatotcytes.

Design: Human PBMC's and hepatocytes were exposed to 10 µM [2',3',8- ^{3}H]ß-L-ddA. At the selected time points, cells were pelleted and extracted overnight with 60% methanol at -20°C. Cells extracts were dried and reconstituted in water then analyzed by HPLC.

Results: ß-L-ddA was found to be not phosphorylated to its 5'-triphosphate at any time point in primary human PBMC's and hepatocytes. In contrast, we detected the presence of 5 metabolites which were identified as R(-)-dihydro-5-(hydroxymethyl)-2(^{3}H)-furanone, hypoxanthine, inosine, ADP, and ATP, with ATP being the predominant reaching levels as high as 8.15 ± 2.64 pmoles/10^6 cells after 4 hrs in human PBMC cells, and 6.43 ± 0.78 pmoles/10^6 cells at 4 hrs in hepatocytes. Inosine, ADP, Hypoxanthine and R(-)-dihydro-5-(hydroxymethyl)-2(^{3}H)-furanone reached lower concentrations. In addition, a ß-glucuronic derivative of ß-L-ddA was also observed in human primary cultured hepatocytes.

Conclusion: This metabolite pathway demonstrated that the lack of antiviral activity of L-ddA is not dependent of its substrate affinity for cellular kinase but involves the breaking of the glycosidic bond of ß-L-ddA followed by a recycling of the base through the de novo salvage pathway leading to formation of endogenous 5'-phosphorylated metabolites of adenosine. This work further emphasizes the need of evaluating each nucleoside analogue currently being tested for its antiviral activity as a unique entity.

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Pre-clinical Pharmacology of ß-L-2',3'-Dideoxy-5-Fluoro-Cytidine and its Prodrug Bis-[(t-butyl)SATE]-ß-L-2',3'-Dideoxy-5-Fluorocytidine Monophosphate. L.T. Martin,[1] A. Faraj,[1] R. F. Schinazi,[2] J.-L. Imbach,[3] G. Gosselin,[3] H. M. McClure,[4] and J.-P. Sommadossi.[1] Univ. of Alabama at Birmingham, Dept. of Clinical Pharmacology, Birmingham, AL 35294, USA;[1] VA Medical Center, Emory Univ., Decatur, GA 30033, USA;[2] Laboratory of Bioorganic Chemistry, Univ. of Montpellier II, Montpellier, Cedex 5, France,[3] Yerkes Regional Primate Research Center, Emory Univ., Atlanta, GA 30322, USA.[4]

The unnatural nucleoside analog, ß-L-2',3'-dideoxy-5-fluorocytidine (ß-L-FddC) possesses potent anti-HIV-1 and anti-HBV activity, *in vitro*. Our group has also demonstrated significantly higher intracellular ß-L-FddCTP level associated with an extended intracellular elimination half-life when compared with its corresponding ß-D-enantiomer. These levels are consistent with the enhanced anti-HIV and anti-HBV activity of ß-L-FddC when compared with ß-D-FddC. The prodrug of ß-L-FddCMP, bis[(t-butyl)SATE]-ß-L-FddCMP (L-FddCMP-SATE) exhibited at least a 5-fold increase in anti-HBV and anti-HIV activity, *in vitro*.

After exposure of 10 µM L-FddCMP-SATE for 4 h to Hep-G2 cells, a high intracellular ß-L-FddCTP level of 113±29 pmol/10^6 cells was detected which was approximately 5-fold greater than that obtained with ß-L-FddC under identical conditions. Intracellular delivery of ß-L-FddCMP via its prodrug was extremely rapid with maximum intracellular ß-L-FddCMP of 141±17 pmol/10^6 cells after a 1h incubation with cells. ß-L-FddCMP levels rapidly declined to 34±7 pmol/10^6 cells by 8 h, but this level was sustained up to 24 h. Exposure of 10 µM L-FddCMP-SATE to stimulated primary cultured human hepatocytes led to rapid intracellular accumulation of the 5'-mono, -di, and triphosphate derivatives of ß-L-FddC. The predominant metabolite, ß-L-FddCTP, achieved intracellular levels of 399±80 pmol/10^6 cells after 4 h of incubation with cells.

Preliminary *in vivo* metabolism studies in rhesus monkeys revealed important hepatic first pass metabolism. Following a 3 mg/kg dose, 57±6% of administered radioactivity was recovered in urine within 48h. Metabolic clearance accounted for 100% of prodrug clearance. Subsequent hepatic extraction and biliary excretion of catabolites was evidenced by positive fecal wipe test. Urine recovery of radioactivy after intravenous and oral administration of [^{3}H]-L-FddCMP-SATE indicated an approximated 82% oral bioavailability while only 45% oral bioavailability was detected after administration of ß-L-FddC. In conclusion, these data demonstrate rapid and efficient intracellular delivery of ß-L-FddCMP *in vitro* by its prodrug, L-FddCMP-SATE and accumulation of higher intracellular triphosphate levels in cells. The *in vivo* metabolism of the prodrug is consistent with *in vitro* metabolism, and most importantly, SATE-prodrugs lead to increased bioavailability of the parent nucleoside.

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Pharmacokinetics of 2',3'-Dideoxy-3'-Fluoro-Guanosine (FLG) in Cynomolgus Monkeys and Rats.

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FLG is a nucleoside analogue which inhibits the replication of hepatitis B and HIV *in vitro* and *in vivo*. The basic pharmacokinetic properties of FLG in cynomolgus monkeys were determined. FLG was given to four monkeys iv (3 mg/kg) and to three monkeys by oral gavage (10 mg/kg). Plasma and urine samples were collected at intervals and analysed by an HPLC method. FLG exhibited a plasma half-life of 27 minutes after i.v dosing and an oral availability of 4%. Microdialysis probes were used to study protein binding which was found to be negligible. Clearance, volume of distribution and mean residence time were all in the range expected for a nucleoside analogue. In rats, the oral availability of FLG was 11%. In an ongoing experiment, FLG has been found to penetrate the blood brain barrier. Prodrugs of FLG with high oral bioavailability have been made making FLG an interesting compound for further development.

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Studies of FLG as a Potent and Selective Inhibitor of Hepatitis B Virus Replication *in vitro* and *in vivo*.

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FLG (2′,3′-dideoxy-3′-fluoroguanosine) is a nucleoside analogue, which inhibits duck hepatitis B virus in primary duck hepatocytes with an IC_{50} of 0.05 μM. Comparative *in vitro* experiments have shown FLG to be 10-fold more active than 3TC and penciclovir. Inhibition of cell growth was observed at 200 μM. FLG was also inhibitory to HIV in cell culture with an IC_{50} of around 2 μM. Resistance development was slow and resulted in the HIV RT mutant 184V. FLG has been evaluated in DHBV-infected ducklings. Inhibition of DHBV-DNA in serum was seen at dosages down to 0.3-1 mg/kg/day. FLG was highly effective when evaluated in SIV-infected monkeys, a model for HIV infection. Toxicity studies in mice, ducks and cynomologus monkeys did not show any significant adverse effect during or after the treatment period 4+4 weeks. Studies in cynomolgus monkeys showed an oral bioavailability of FLG of 4 % and a oral plasma half-life of 1.2 hours. Synthesis of new prodrugs of FLG have improved the oral bioavailability to about 50%. An FLG prodrug has been scheduled for clinical development.

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Anti-DHBV and -SIV Activity of 2',3'-Dideoxy-3'-Fluoro-Guanosine (FLG) *in vivo*.

H. Ageland[1], C. Åhgren[1], R. Benthin[1], D. Böttiger[1], N-G. Johansson[1], B. Löfgren[2], B. Öberg[1,3], M. Pelcman[1], C. Rydergård[1], I. Schröder[2], X-X. Zhou[1].

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FLG (2',3'-dideoxy-3'-fluoro-guanosine) is a nucleoside analogue active against hepatitis B virus (HBV) and human immunodeficiency virus (HIV). In cell culture FLG is more active against duck hepatitis B virus (DHBV) than 3TC and PCV, but less active than AZT against HIV and simian immunodeficiency virus (SIV). FLG was evaluated for antiviral effect *in vivo* against SIV and DHBV. FLG (3x5 mg/kg for five days, sc) prevented SIV infection in two out of four cynomolgus monkeys and delayed the appearance of viral antigen in the other two monkeys compared to the controls. Eleven of twelve control animals became infected. At 3x15 mg/kg AZT had a limited effect in delaying SIV antigen appearance in this model. Congenitally DHBV-infected ducklings were treated i.p. with FLG at 0.3-5 mg/kg for ten days and DHBV DNA levels in plasma were reduced. When treatment was stopped the virus reappeared. FLG showed a pronounced antiviral effect *in vivo* against SIV and DHBV which together with other properties make it a candidate for further development.

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Anti-Hepatitis B Virus Activity of 3-Hexadecyl-oxypropane-1-phospho-penciclovir and 3-Hexa-decyloxypropane-1-phospho-dideoxyguanosine in 2.2.15 Cells.

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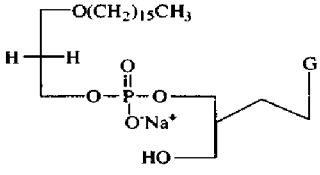
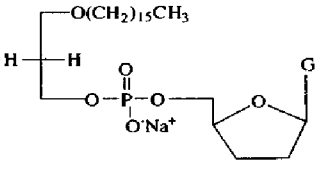
A recent report from our laboratory has demonstrated that a phospholipid prodrug of acyclovir (ACV), 3-hexadecyloxypropane-1-phospho-acyclovir (HDP-P-ACV) shows significant enhancement of antiviral activity against HBV, in 2.2.15 cells, in vitro in comparison with



HDP-P-PCV



HDP-P-ddG

ACV. We have now synthesized lipid prodrugs of the two purine nucleoside analogs, penciclovir (PCV) and 2',3'-dideoxyguanosine (ddG). In vitro evaluation of HDP-P-PCV against HBV in 2.2.15 cells indicated the prodrug to be 3-fold more active than PCV. HDP-P-ddG exhibited a similar increase in potency as compared to ddG. These results, as well as the anti-HIV activity of the prodrugs will be presented.

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Phospholipid Prodrugs of Antiviral Nucleosides: Efficacy of Orally Administered 3-Hexadecyloxy-propane-1-phospho-acyclovir Against Hepatitis B Infection in Woodchucks.

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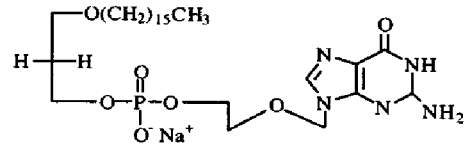
We reported recently (Biochem. Pharmacol., 53: 1815 - 1822, 1997) that phospholipid prodrugs of acyclovir (ACV) exhibit greatly increased in vitro anti-HBV activity versus ACV in stably transfected hepatoma cells (2.2.15), and that these conjugates are orally bioavailable in mice. We have now evaluated the activity of hexadecyloxypropane-phospho-acyclovir (HDP-P-ACV) in the woodchuck hepatitis B virus (WHBV) model. The oral administration of HDP-P-ACV (10 mg/kg bid, 28 days) to WHBV-infected woodchucks induced a 96 % reduction of viremia levels as compared to untreated control animals. Oral administration of acyclovir (20 mg/kg bid, 28 days) did not inhibit WHBV replication. These results confirm the in vitro data and show that the alkoxypropane-phosphate prodrug of acyclovir is orally active in vivo.



HDP-P-ACV

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

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

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Inhibition of DHBV DNA Replication with Antisense Oligodeoxynucleotides in Vitro and in Vivo, PZ Tao, B Dong, XW Shao, Institute of Medicinal Biotechnology, CAMS, Beijing, P.R.China

Three antisense oligodeoxynucleotides have been synthesized as phosphorothioate analogues which correspond to duck hepatitis B virus (DHBV) PreS1, PreS2 and S antigen gene promoter respectively. In primary duck hepatocyte culture 1.5uM of these antisense oligodeoxynucleotides displayed inhibitory effects on DHBV DNA replication with the inhibition rates of 61.5%, 69.3% and 62.4% respectively. In vivo DHBV infected ducklings were treated by 10ug/g PreS1 antigen gene promoter antisense oligodeoxynucleotide per day for 6 days. The medication could inhibit DHBV DNA replication by 81.4% with the method of Southern blot and cpm calculation. These results demonstrated the potential clinical application for antiviral therapy of antisenseoligodeoxynucleotides in the future.

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Development of a Moderate Throughput Assay Using TaqMan PCR Technology to Identify Inhibitors of Hepatitis B Virus Halliday, S.M., Pallansch, L.A., and Buckheit, R.W., Jr., Southern Research Institute, Frederick, MD, USA

We have developed a microtiter-based, moderate throughput assay to identify inhibitors of HBV *in vitro*. This assay utilizes an immortalized cell line transfected with multiple copies of the HBV genome (HepG2 2.2.15). Real time detection is performed using TaqMan PCR technology, in which a fluorogenic probe hybridizes to the target of interest and results in the generation of a fluorescence signal proportional to the amount of product present. We have been able to screen up to 100 compounds per day for the identification of new active leads utilizing this assay system. The nucleoside analogs 3TC and ddC have been previously reported as inhibitors of HBV infection *in vitro*. A comparison of effective concentrations of these compounds determined in our assay and those determined in previously published reports is presented here. In addition, a series of nucleoside analogs was evaluated for anti-HBV activity in this assay system. Effective concentrations and structures of several of these compounds are presented.

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Mutations in Human Hepatitis B Virus Polymerase Associated with Resistance to Lamivudine and Famciclovir Do Not Decrease Sensitivity to Adefovir. X. Xiong, C. Flores, H. Yang, C.E. Westland, J.J. Toole, and C.S. Gibbs. Gilead Sciences, Foster City, CA, USA.

Adefovir (PMEA) is an acyclic nucleotide analog with broad spectrum antiviral activity against retroviruses, hepadnaviruses and herpesviruses. adefovir dipivoxil (the oral prodrug of PMEA) is in clinical trials for the treatment of HBV and HIV infections. HBV DNA polymerase mutations at M552I and, more commonly, at L528M/M552V have been observed in HBV patients that developed resistance to lamivudine (3TC). The L528M mutation also has been found in HBV patients that developed resistance to famciclovir. To determine whether lamivudine-, or famiciclovir-resistant mutants are cross-resistant to adefovir, we compared the sensitivity of wild-type and mutant HBV DNA polymerases (M552I, M552V, L528M, and L528M/M552V) to the activated forms of adefovir (PMEApp) and lamivudine (3TCTP). We overexpressed and substantially purified the wild type and the mutant human HBV DNA polymerases using a baculovirus expression system and immuno-affinity chromatography. Inhibition constants were determined by an *in vitro* HBV DNA polymerase assay.

	Fold Increase (Ki mutant/Ki wild type)			
	M552I	M552V	L528M	L528M/M552V
PMEApp	2.8	2.2	2.3	0.79
3TCTP	7.6	19.6	2.6	20.4

Consistent with the clinical data, we found that M552I, M552V, and L528M/M552V mutations exhibited inhibition constants increased by 8 to 20-fold for 3TCTP compared to that of wild type HBV DNA polymerase. However, these mutants remained sensitive to adefovir with the inhibition constants changing by only 0.8 to 2.8-fold compared to the wild type. The L528M mutation, associated with famciclovir-resistance, also remained sensitive to adefovir. We are closely monitoring HBV genotypical changes in chronic hepatitis patients treated with adefovir dipivoxil in ongoing Phase II clinical trials. No mutations have been found in the HBV polymerase/RT domain in the first four samples obtained from patients treated with 30 mg adefovir dipivoxil daily for 12 weeks.

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Selection of Woodchuck Hepatitis Virus Resistant to 3TC due to a Mutation in the Highly Conserved Polymerase FLLA Region in a Lifetime Woodchuck Antiviral Trial.

Schinazi, R. F.,[1*] Stang, H.L.,[1] Tatti, K.M.,[1] Korba, B. E.,[2] S. Peek,[3] and Tennant, B.C.[3] Lab. Biochem. Pharmacol., Dept. of Pediatrics, VAMC/Emory Univ., Decatur, GA 30033;[1] Mol. Virol. and Immu., Georgetown Univ., Rockville, MD 20852;[2] and Coll. of Vet. Med., Cornell Univ., Ithaca, NY 14853.[3]

We recently demonstrated that treatment with 3TC initiated early in the course of chronic WHV infection significantly inhibited hepatocarcinogenesis in a lifetime antiviral trial in chronically infected woodchucks. Since virus recrudescence in serum of some woodchucks treated with 3TC occurred, we examined twelve coded woodchuck plasma samples, six control samples and six treated with 3TC, obtained at 80 weeks. Of the six 3TC treated samples, three came from woodchucks with high levels of viral recrudescence in plasma as determined by Southern blotting. A DNA fragment containing domains A-E of the polymerase was isolated by PCR for each sample and analyzed by automated nucleotide sequencing. Surprisingly, all twelve samples were wild-type at the conserved YMDD motif in domain C associated with 3TC resistance in HBV. Four of the six 3TC treated woodchuck samples showed a mixture of the wild-type A (GCT) and the mutant T (ACT) at the conserved amino acid residue 566 (FLLA) in domain B. Interestingly, this change is associated with the amino acid 526 (FLLA) in HBV where 3TC and famciclovir selects for a change from L to M. In the woodchuck, the A to T change in the *pol* region results in a mutation of the surface protein from amino acid T (TGG) to an opal codon (TGA) which terminates the polypeptide. This transitional mutation creates a truncation in the surface protein forming a product reduced by 54 amino acids. The significance of these findings in relation to HBV pathogenesis and drug-resistance will be discussed.

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Effects of HPMPC on Inhibition of HPV-16 Transformed Cell Proliferation

J. A. Johnson* and J. D. Gangemi

Department of Microbiology and Molecular Medicine and the Greenville Hospital System Biomedical Cooperative, Clemson University, 445 Brackett Hall, Clemson, SC 29634

(S)-1-(3-hydroxy-2-phosphonylmethoxy-propyl)cytosine (HPMPC) is a nucleoside phosphonate analog known to inhibit herpesvirus DNA polymerase. We have demonstrated that this compound can suppress proliferation of cells infected with human papillomavirus (HPV), which does not possess a viral DNA polymerase. We have shown that HPMPC induces an apoptotic response in HPV-16 transformed cells, indicating that this analog is cytotoxic rather than cytostatic. In order to further elucidate the mechanism of cell growth inhibition, we measured changes in cell cycle indicator/regulator expression and thymidine incorporation following treatment with HPMPC. HPMPC treatment reduced WAF1 (p21) levels, independently of p53, while augmenting expression of proliferating cell nuclear antigen (PCNA). However, in comparison to controls, HPMPC treated cells display a decrease in thymidine incorporation, indicating an inhibition of host DNA polymerase activity. Cell cycle analysis has revealed that HPMPC treated HPV-transformed cells are driven from G1 into the S-phase, but do not continue onto G2/M. The data suggest that an apoptotic response is initiated in HPV transformed cells trapped in S-phase.

100

Regulation by Human Papillomavirus E2 Proteins and Applications to Antiviral Drug Discovery

R. Kovelman[1], G. K. Bilter[1], E. Glezer[1], A. Roman[2], D. R. Brown[3], and M. S. Barbosa[1]

[1]Virology Group, Signal Pharmaceuticals, Inc., San Diego, CA; [2]Department of Microbiology and Immunology, Indiana University School of Medicine, and The Walther Cancer Institute, Indianapolis, IN; [3]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN.

Human papillomaviruses (HPVs) cause anogenital warts and other epithelial diseases and are linked to cervical cancer. The HPV E2 gene encodes a DNA-binding transcription factor. A systematic comparison of transcriptional activation by HPV E2 proteins revealed that the E2 proteins from HPVs linked to cervical cancer ("high-risk" HPV-16 and HPV-18) are much more active than the E2 proteins from low-risk HPVs (HPV-6 and HPV-11). *In vivo* experiments using a number of different cell types as well as *in vitro* assays demonstrated that this difference was intrinsic to the proteins and did not result from divergent DNA-binding properties or altered protein levels. In order to determine whether the lower observed transcriptional activity of the E2 proteins from low-risk HPVs was a consequence of the particular HPV-6 and HPV-11 clones being used, we also analyzed the sequence of E2 coding regions in HPV-6-containing clinical isolates and the activity of the encoded proteins. We found that these E2 proteins, the sequences of which fell into two categories, were also of low activity. Our studies thus demonstrate that there are at least two functional categories of HPV E2 protein, and this categorization needs to be taken into account when designing antiviral assays using the E2 protein as a target.

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Effect of Acyclic Nucleoside Phosphonates (ANPs) on the Proliferation of Adenovirus-transformed Cell Lines as Compared to Human Papillomavirus (HPV)-harboring Cell Lines

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Rega Institute for Medical Research, K.U.Leuven, B-3000 Leuven, Belgium

We have shown that treatment with ANPs of cell lines harboring HPV resulted in a time-dependent inhibition of cell proliferation. This inhibitory effect is particularly more striking for cidofovir (HPMPC). A 34- to 75-fold decrease in the 50% cytotoxic concentration (CC_{50}) at day 7 (as compared to day 3) was observed with HPMPC for several HPV-positive cell lines. A 5- to 15-fold decrease in CC_{50} was noted for HPMPC at day 7 in tumor cell lines non-containing HPV, but not in normal human cells. These results suggest that the anti-HPV activity of HPMPC may be explained, at least in part, by an anti-proliferative effect on rapidly proliferating cells and HPV might enhance the sensitivity of the cells to HPMPC due to an interaction of the viral transforming proteins with products of tumor suppressor genes. We have now evaluated the effects of the ANPs and other antiviral and antitumor drugs on SV_{40}-transformed African green monkey kidney (COS-1 and COS-7) cells and adenovirus-transformed human embryo kidney (293) cells, since viral transforming proteins of these cells are also known to interact with products of tumor suppressor genes. Treatment of COS-1, COS-7 and 293 cells with ANPs resulted in inhibition of cell proliferation in function of time similar to that observed for HPV-positive cells. The CC_{50} of HPMPC varied from 14-22 µg/ml at day 3 to 0.55-0.88 µg/ml at day 7, while the CC_{50} of HPMPC for the corresponding non-transformed cells varied from 92 µg/ml (Vero cells, African green monkey kidney) and 128 µg/ml (HEL cells, human embryonic lung fibroblasts) at day 3 to 12 µg/ml (Vero cells) and 84 µg/ml (HEL cells) at day 7. These resulted in a selectivity index (ratio CC_{50} for non-transformed cells to CC_{50} for transformed cells at day 7 of 16 (COS-1 cells), 18 (COS-7 cells) and 95 (293 cells). The effect of ANPs on the interaction of viral transforming proteins with products of tumor suppressor genes is currently under investigation.

Oral Session VI: Herpesvirus Infections II

102

Inhibition of Human Cytomegalovirus Protease N_o with Monocyclic β-Lactams.

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Human cytomegalovirus, like all the herpesviruses, encodes a unique serine protease which is necessary for viral replication. The recent determination of the X-ray structure of the HCMV N-terminal protease domain (N_o) of gene UL80 revealed that this enzyme belongs to a novel class of serine proteases. The active site is composed of a triad consisting of His63, His157 and Ser132. Since their discovery, herpesvirus proteases have become attractive molecular targets for the design of novel antiviral drugs. We recently reported a series of substrate-based activated carbonyl inhibitors of the title protease in which we described a peptidic β-lactam derivative (Ogilvie *et al*, J. Med. Chem **1997**). We now report a new series of non-peptidic monocyclic β-lactam inhibitors. The structure-activity relationship work that led to the discovery of these small, potent and selective molecules will be disclosed. The mechanism of inhibition of the HCMV protease by these new β-lactams will also be discussed.

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Discovery of A Novel Class of Potent Heterocyclic Human Cytomegalovirus Inhibitors and Their Structure-Activity Relationship Studies.

H. Jin, L.Chan, T. Stefanac, W. Wang, J-F. Lavallée, G.Falardeau, J. Bedard, S. May and L. Yuen. BioChem Therapeutic Inc., Laval, Québec, Canada H7V 4A7

All the currently available anti-HCMV agents on the market are based on inhibiting HCMV DNA polymerase with various undesirable toxicity and poor oral bioavailability. Development of orally bioavailable HCMV inhibitors with no serious side effects is a primary objective facing the pharmaceutical industry. Through our extensive screening program, we identified a completely novel class of HCMV inhibitors based on standard plaque reduction assay in human fibroblast cells. The lead compound inhibits HCMV replication at submicromolar concentration with high selectivity index. Following the focused structure-activity relationship studies, we found a specific structural requirement for the activity in these naphthyridine and isoquinoline analogs. A few compounds emerged with anti-HCMV activity in the low nanomolar range with high selectivity index. Moreover, in our assays, these compounds are more potent than Ganciclovir, Cidofovir and 1263W94 with equal or less cytotoxic effects. Preliminary *in vitro* results suggest that these compounds exert antiviral activity at an early stage of viral infection with a possibly novel mechanism.. Our findings indicate that these compounds merit further investigation to be developed into clinically useful HCMV therapeutics.

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The Furin-Directed α_1-Antitrypsin Variant α_1-PDX: A Potent New Anti-Cytomegalovirus Drug. François Jean, Laurel Thomas, Jay Nelson[1] and Gary Thomas. Vollum Institute and [1]Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR, USA.

Many viruses require processing of their envelope proproteins at the consensus furin site (-Arg-X-X-Arg-). Efforts to block the key enzyme(s) mediating this cleavage resulted in the generation of various inhibitors directed against the proprotein convertase furin. Cellular expression of a furin-directed α_1-antitrypsin variant, α_1-PDX (reactive site loop mutated to -Arg-X-X-Arg-to inhibit furin) blocks the processing of HIV-1 gp160 and Measles virus F_0. Importantly, inhibition of viral envelope glycoprotein processing by α_1-PDX greatly suppresses the production of infectious virus [Watanabe *et al.*, (1995) J. Virol. **69**:3206]. Similar to HIV-1 and Measles virus, infectivity of HCMV requires cleavage of its envelope glycoprotein gB at a consensus furin site. Furthermore, when co-expressed in cells, α_1-PDX blocks the maturation of gB. To explore the usefulness of this protein as an antiviral agent, a His- and FLAG- tagged α_1-PDX, α_1-PDX/hf, was constructed and expressed in bacteria. Treatment of HCMV-infected human glioma (U373) cells with 20 μM α_1-PDX/hf resulted in a three-log decrease in the titer of infectious cell-associated HCMV (ED_{50} = 8 μM). By contrast, 300 μM of the antiviral drug phosphonoformic acid (PFA; foscarnet) (ED_{50}: 80 μM) was necessary to observe the same effect. Furthermore, no toxic effect of the α_1-PDX/hf was detected in cultured cells at the concentrations tested (> 40 μM). These results, as well as the mechanism by which extracellularly applied α_1-PDX/hf inhibits intracellular furin, will be presented. This work was supported in part by NIH. F. Jean is an MRC post-doctoral fellow.

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Benzimidazole L-Sugar Nucleosides: Large Biological Changes from Small Structural Modifications. J.C. Drach, M.T. Migawa, J.-L. Girardet, R.G. Ptak, C. Oh, S. Barnat, J.A. Walker II, R.V. Devivar, S.D. Chamberlain,† G.W. Koszalka,† M.R. Underwood,† K.K. Biron,† and L.B. Townsend. University of Michigan, Ann Arbor, Michigan 48109 and Glaxo Wellcome Co., Research Triangle Park, NC 27709†, U.S.A.

We have previously described the synthesis and antiviral activity of the 2,5,6-trihalobenzimidazole ß-D-ribosides TCRB and BDCRB (Townsend *et al., J. Med. Chem.* **38**:4098-4105, 1995) and the ß-L-riboside 1263W94 (Koszalka *et al., Antiviral Res.* **30**:A43, 1996). Both these D- and L-isomers are active against human cytomegalovirus (HCMV) but the former inhibit viral DNA processing whereas the latter inhibits viral DNA synthesis. We now report the synthesis and biological activity of α-L-arabinofuranosyl and α-L-5′-deoxylyxofuranosyl trihalobenzimidazoles which can be considered, respectively, as 3′,4′-epimers of TCRB or 4′-epimers of the 5′-deoxy analog of TCRB (5′-dTCRB). Trihalobenzimidazoles of the former series were weakly active against HCMV and were not cytotoxic at 100 μM. In contrast, the 2-Cl and 2-Br analogs in the α-L-5′-deoxylyxofuranosyl series (compounds 1311, 1325) were much more active against HCMV (IC_{50} = 0.2 - 0.4 μM) with cytotoxicity IC_{50}'s = 30 - 60 μM. Based on viral DNA synthesis in a single cycle assay, activity in benzimidazole resistant strains of HCMV, and BDCRB activity reversal experiments, 1311 appeared to act by inhibition of viral DNA synthesis. This was surprising because the α-L-5′-deoxylyxofuranosyl nucleosides are 4′-epimers of 5′-dTCRB which acts by inhibition of DNA processing (Drach *et al.* 35th ICAAC, San Francisco, CA, 9/95). Thus minor changes in the spatial orientation of the 3′- and 4′-positions of the sugars of benzimidazole nucleosides radically affect not only HCMV activity but also mode of action. These studies were supported by research grant UO1-AI31718 from NIAID and Research Agreement DRDA-942921 with Glaxo Wellcome Co.

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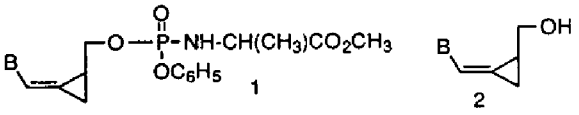
PHOSPHORALANINATE PRODRUGS OF METHYLENECYCLOPROPANE ANALOGUES OF NUCLEOSIDES AS ANTIVIRAL AGENTS. J. Zemlicka[1], Y.-L. Qiu[1], J.-S. Lin[2], Y.-C. Cheng[2], R. G. Ptak[3], J. M. Breitenbach[3], J. C. Drach[3] and E. R. Kern[4], [1]Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201; [2]Yale University School of Medicine, New Haven, CT; [3]School of Dentistry, University of Michigan, Ann Arbor, MI; [4]University of Alabama School of Medicine, Birmingham, AL.

The title compounds 1a - 1c were synthesized and their antiviral activity against HCMV, HSV-1, HSV-2, EBV, VZV, HIV-1 and HBV was investigated. Adenine analogue 1a was more potent against all these viruses than synadenol (2a) but this effect was accompanied by a varying



Series a: B = Ade; series b: B = Gua;
series c: B = 2,6-diaminopurine

degree of cytotoxicity. In HIV-1/CEM-SS assay EC_{50} of 1a was 0.05 μM and CC_{50} 1.1 μM. Guanine derivative 1b was less active against HCMV/HFF than synguanol (2b) with EC_{50} 4.2-7.6 μM and CC_{50} >100 μM but more potent toward HBV/2.2.15 (EC_{50} 2 μM, CC_{50} >50 μM). Most interesting is the 2,6-diaminopurine analogue 1c which was very effective against HCMV/HFF, HIV-1/CEM-SS, HBV/2.2.15, EBV/Daudi, EBV/H-1 and VZV/HFF with EC_{50} 4-11, 0.2, 0.08, 3.8, 5.5 and 1.1 μM, respectively. The corresponding CC_{50}'s were 211 (HFF), 35 (CEM), 45 (2.2.15) and 26 (Daudi). Parent compound 2c exhibited a significant activity only against EBV. Possible reasons for a high *in vitro* efficacy and low cytotoxicity of 1c which contains an unnatural nucleic acid base will be presented. Analogue 1c effective against five different types of viruses, HCMV, HIV-1 and HBV, EBV and VZV at non-cytotoxic concentrations, is a subject of further studies. Supported by NIH grants RO1-CA32779, RO1-AI33655, RO1-38204, U19-AI31718, RO1-AI33332 and NO1-AI-35177.

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Inhibition of Human Cytomegalovirus Replication in SCID-hu Retinal Tissue Implants by Ganciclovir and Cidofovir. D.J. Bidanset, R.J. Rybak, C.B. Hartline, and E.R. Kern. University of Alabama School of Medicine, Birmingham, AL.

Human Cytomegalovirus (HCMV) infections cause a wide range of clinical manifestations, especially in the immunocompromised host. Presently, there are few models to study HCMV infection since virus infection and replication is largely limited to human cells. In this study, we present a model for utilizing HCMV infection of fetal human retinal tissue implanted in the eye of severe combined immunodeficient (SCID) mice. Small fragments of fetal human retinas were implanted into the anterior chamber and 1 to 2 wks later inoculated with 1000-5000 pfu of HCMV. At various times after infection, animals were sacrificed, eyes were enucleated and homogenized in 1.0 ml of medium, and HCMV titers were determined by plaque assay. The results indicated that HCMV replicated in the implanted tissue and titers increased 2-3 logs by 28 days post infection then gradually decreased to undetectable levels by day 70. Treatment of mice, i.p., with 45 mg/kg ganciclovir twice daily for 2 wks followed by once daily for 2 wks resulted in a 30-fold decrease in virus titer. However, 2 wks after cessation of treatment, the virus titer began to gradually increase. Treatment of mice with 25 mg/kg cidofovir once daily for 5 days followed by twice weekly through day 28 resulted in a 20-fold decrease in virus titer by day 14 which gradually increased to only a 2-fold decrease by day 28. These data indicate that this model of infection may be useful for determining the efficacy of various antiviral therapies against HCMV infection in ocular tissue.

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Mutations Occur in Highly Conserved Domains of Murine Cytomegalovirus DNA Polymerase in Cidofovir- and Lobucavir-resistant Strains. K.M. TATTI,[1]* H. STANG,[1] D. BARNARD,[2] D. SMEE,[3] and R. F. SCHINAZI.[1] Emory University School of Medicine and VA Medical Center, Decatur, Georgia;[1] Utah State University, Logan, Utah,[2] and USAMRID, Fort Detrick, Frederick, MD.[3]

Many HIV-infected individuals are coinfected with human cytomegalovirus (HCMV) which can lead to retinitis and other complications. Since the development of resistant virus has been observed with currently approved anti-CMV agents, new agents to treat this infection are needed. Murine cytomegalovirus (MCMV) has close genetic similarity to HCMV and can be used in mice to evaluate antiviral agents against drug-resistant viruses. We selected cidofovir (HPMPC)- and lobucavir (CBG)-drug-resistant MCMV strains in murine cells. Cross-resistance patterns, phosphorylation, and sequencing studies using these resistant strains indicated that the viruses contained mutations in the viral DNA polymerase gene. Nucleotide sequencing of the entire polymerase gene from the CBG-resistant strain confirmed that a highly invariant Thr at amino acid 743 in the conserved domain, region III, was found to be mutated to an Ala. For the HPMPC-resistant strain, a mutation at amino acid 726 had occurred (Thr to Met) also in region III, a domain which is involved in substrate binding. In addition, the HPMPC-resistant strain contained two additional mutations in region A of the DNA polymerase gene. Site-directed mutagenesis studies should confirm the relevance of these mutations in the observed resistant phenotype *in vitro* prior to planned studies in animal models. *(Supported by NIH grant AI-39378 and the VA)*

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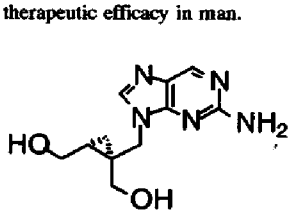
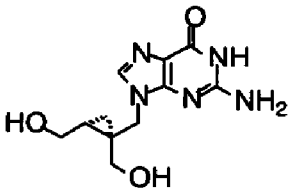
Pharmacokinetics and Efficacy of AV-038, an Oral Prodrug of a Novel Potent Antiherpetic Nucleoside Analogue A-5021. S. Iwayama, K.Suzuki, T. Nakagawa, T. Sekiyama, T. Ohnishi, Y. Ohmura, N. Ono, H. Nakazawa, O. Ikemura, T. Tsuji, M. Okunishi. Ajinomoto Co., Inc., Kawasaki, Japan.

A-5021, (1'*S*,2'*R*)-9-[[1',2'-bis(hydroxymethyl)cycloprop-1'-yl]methyl]guanine, is a guanosine analogue possessing superior antiviral activity over ACV and PCV against HSV-1, HSV-2, VZV and HCMV. However, the oral bioavailability of A-5021 is limited like other guanosine derivatives. During a search for an oral prodrug of A-5021, we found that the 6-deoxy derivative of A-5021 (AV-038) had a good oral absorption in mouse, rat, dog, and monkey. However, there was interspecies difference in the bioavailability of A-5021 after oral administration of AV-038 being highest in monkey (approximately 70%) and lowest in dog (approximately 4%). To estimate the conversion of AV-038 to A-5021 in human, in vitro study using human liver slice was conducted and it was revealed that AV-038 is efficiently converted to A-5021 at the comparable rate of the conversion of 6-deoxy penciclovir, the deacetylated form of famciclovir, to penciclovir. In mice infected with HSV-1, orally administered AV-038 showed an efficacy consistent with its oral bioavailability of A-5021. These findings show the possibility that AV-038 will be converted rapidly to A-5021 and show good therapeutic efficacy in man.



AV-038



A-5021

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In Vivo Anti-CMV Activity and Safety of Oral 1263W94 in HIV-Infected Subjects with Asymptomatic CMV Shedding. DREW WL, LALEZARI JP, WANG LH, MINER RC, ABERG JA, WIRE MB, JACOBSON MA. UCSF Mount Zion Medical Center and Quest Clinical Research, San Francisco, CA; Glaxo Wellcome Inc., Research Triangle Park, NC; UCSF General Hospital, San Francisco, CA.

1263W94, a benzimidazole riboside, has demonstrated potent anti-CMV activity *in vitro* and good safety and pharmacokinetic profiles in Phase I trials. A Phase I/IIa trial has been conducted to evaluate the *in vivo* anti-CMV activity and safety of 1263W94 at doses of 100, 200 and 400 mg tid for 28 days in male HIV-infected subjects with asymptomatic CMV shedding. In the open-label subset of the study, 19 enrolled subjects (N=6-7 per dose) had semen CMV titer (by plaque assay) ranging from 3.6 to 6.7 $\log_{10}$ PFU/mL and positive CMV urine culture at study entry. None of these subjects had CMV disease or positive blood CMV culture and their CD4+ cell counts ranged from 34 to 420 c/μL. Antiviral activity was measured by reduction of CMV titer in semen and eradication of CMV viruria. There were no serious adverse events and no clinically significant laboratory abnormalities. The most commonly reported adverse events were taste disturbance, fatigue, headache, and nausea. 1263W94 showed dose-dependent increase in the rate and extent of CMV titer reduction in semen and an anti-CMV activity in urine (culture conversion from ++ at baseline to -):

Dose (mg tid)	Day 14		Day 28	
	Semen Titer Δ ($\log_{10}$)	Urine (% ++ to -)	Semen Titer Δ ($\log_{10}$)	Urine (% ++ to -)
100	-1.1	40%	-2.9	40%
200	-1.6	50%	-3.7	75%
400	-2.8	60%	-3.7	60%

The decreases in semen titer were minimum estimates since 71, 80 and 100% of subjects, respectively, had semen CMV titer drop below limit of quantitation (25 PFU/mL) on Day 28 at each escalating 1263W94 dose. The favorable safety profile and encouraging *in vivo* anti-CMV activity of 1263W94 obtained from this ongoing clinical trial support further investigation of this oral anti-CMV agent.

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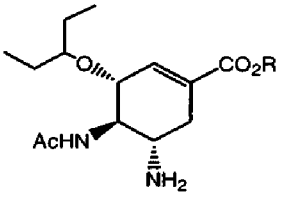
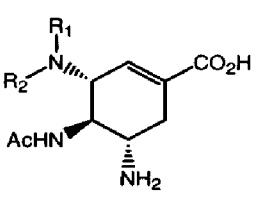
Synthesis and Activity of a New Series of C_3-Aza Carbocyclic Influenza Neuraminidase Inhibitors. W. Lew[1], H.W. Wu[1], D.B. Mendel[1], C.Y. Tai[1], P.A. Escarpe[1], C.U. Kim[1], W.G. Laver[2] and B. J. Graves[3].

[1]Gilead Sciences Inc., Foster City, CA, USA, [2]Australian National University, Canberra, Australia and [3]Roche Products, Ltd., Welwyn Garden City, Hertfordshire, U.K.

We have previously described a new class of potent carbocyclic influenza neuraminidase inhibitors based on a cyclohexene scaffold. In this series, a new hydrophobic pocket in the region corresponding to the glycerol subsite of sialic acid was identified. By exploring the hydrophobic interactions in this active site, compound 1 (GS 4071) emerged as one of the most potent influenza neuraminidase inhibitors. The ethyl ester of 1 (2, GS 4104) is currently being evaluated in clinical trials as an oral agent for the treatment and prophylaxis of influenza infection. As part of our ongoing structure activity relationship studies of this class of compounds the corresponding C_3-aza analogues 3 were prepared. The synthesis and activity of these new C_3-aza analogues will be discussed.



1 R = H (GS 4071)
2 R = Et (GS 4104)



3

Oral Session VII: Respiratory Virus Infections, Emerging Infections

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Lack of In Vivo Development of Influenza A Virus Resistance to the Orally Effective Neuraminidase Inhibitor GS4104.
R.W. Sidwell, K.W. Bailey, M.H. Wong, J.H. Huffman. Inst. for Antiviral Research, Utah State Univ., Logan, UT, USA.

Development of viral resistance to antiviral drugs is a potential public health problem which has been seen in influenza virus-infected patients treated with amantadine or rimantadine. The carbocyclic transition state sialic acid analog GS4071, a potent inhibitor of influenza neuraminidase, is markedly effective against influenza virus in vitro; GS4104, the ethyl ester prodrug of GS4071, has shown striking efficacy against this virus infection in orally treated mice. A study was run to determine if mice infected with influenza A/Shangdong /09/93 (H3N2) virus and treated with 100, 10 or 1 mg/kg/day of GS4104 would produce a GS4071-resistant virus. Treatment was by oral gavage twice daily for 5 days beginning 4 h pre-virus exposure Lungs from 5 mice at each dosage and from placebo-treated controls were taken on day 6, 2 days after termination of treatment, and virus recovered from each was titrated. A 50% cell culture infectious dose of each virus preparation was used in in vitro antiviral experiments with varying doses of GS4071, utilizing inhibition of visually determined cytopathic effect and neutral red dye uptake as endpoint parameters. Treatment with the 100 and 10 mg/kg/day doses of GS4104 was protective to the infected animals; the virus recovered from two of their lungs displayed a 3-fold ($P<0.05$) reduction in sensitivity to GS4071 compared to virus from placebo-treated control animals; all other GS4104-treated mice had no reduced sensitivity. Similar experiments with amantadine and rimantadine demonstrated a 1000-fold reduced sensitivity of influenza virus to these compounds, with virus from all lungs showing similar resistance. (Supported by contract NO1-AI-65291 from the Virology Branch, NIAID, NIH).

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In Vitro Selection and Characterization of a Human Influenza Virus With Decreased Susceptibility to GS 4071. D.B. Mendel, C.Y. Tai, and P.A. Escarpe. Gilead Sciences, Inc., Foster City, CA, USA

GS 4071 (Ro 64-0802) is a novel, potent and selective inhibitor of the influenza neuraminidases. Oral administration of GS 4104 (Ro 64-0796), an ethyl ester prodrug of GS 4071, has demonstrated clinical efficacy and antiviral effect in experimental influenza A infection in humans, and is currently under development for the prophylaxis and treatment of influenza A and B infections in man. To investigate the potential development of resistance to this compound, we have passaged the influenza A/Victoria/3/75 (H3N2) virus in vitro using 2-fold increased concentrations of GS 4071 for each passage. Viral variants containing a mutation at the conserved Arg292 of the neuraminidase gene were detected after 12 passages. The mutant neuraminidase exhibited high level (30,000-fold) resistance to GS 4071, but only low level (30-fold) cross resistance to GG167 and the guanidino derivative of GS 4071. The mutant enzyme was compromised in terms of its enzymatic activity, and the mutant virus, which also contained two hemagglutinin mutations located in regions distinct from the receptor binding pocket, replicated poorly in culture compared to either the wild type virus, or a virus containing only the hemagglutinin mutations. In another study (Gubareva et al., J. Virol. 71:3385, 1997), the infectivity in mice of a virus containing the same Arg→Lys mutation at residue 292 of the neuraminidase was found to be 500-fold lower than the wild type virus, suggesting that this mutation may have limited clinical significance.

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Chemoprophylaxis of influenza A infections in mice and ferrets with neuraminidase inhibitors, exemplified by zanamivir (GG167). Fenton, R.J., Owens, I.J., Morley, P.J. Glaxo Wellcome R&D, Stevenage, Hertfordshire, U.K.

The constant antigenic change and highly infectious nature of influenza viruses means that whole communities are continually at risk of infection. With an antiviral compound indicating a good safety profile, there exists the opportunity to protect susceptible populations by treatment prior to the onset of symptoms i.e. chemoprophylaxis. Influenza neuraminidase inhibitors, typified by zanamivir (GG167) have been shown to be safe and effective antivirals in both animal and human models of influenza virus infection when administered both prophylactically and therapeutically. We further investigated the relationship between time of compound administration and prophylactic efficacy for neuraminidase inhibitors, exemplified by zanamivir. The effect of prophylactic doses was assessed in both mouse and ferret models of influenza A virus infection. A subclinical infection model with A/Singapore/1/57 (H2N2) in the mouse was employed to determine the effect on lung virus titre of single intranasal doses given at various time-points prior to infection. In addition, the effect on lung virus titre of various doses given at fixed time-points prior to infection was studied using this model. Single intranasal doses given prior to infection in mice, significantly reduced lung virus titre compared to vehicle treated controls. In a ferret model of A/Mississippi/1/85 (H3N2) infection, a single intranasal dose given prior to infection reduced both nasal wash virus titres and pyrexia associated with infection compared to vehicle treated controls. These data suggest not only a possible role for influenza neuraminidase inhibitors in the prophylaxis of influenza infections in man, but that dosage interval may be extended. Clinical studies have been initiated to investigate this.

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Potent Inhibition Of Respiratory Syncytial Viruses (RSV) By A 2-5A-Antisense Oligonucleotide Chimera Targeted To Intragenic Signals In The RSV Genome. D. L. Barnard[1†], R. W. Sidwell[1], J. E. Matheson[1], M. Player[2] and P. F. Torrence[2]. [1]Institute for Antiviral Research, Utah State University, Logan, UT, USA and [2]Section of Biomedical Chemistry, NIDDK, NIH, Bethesda, MD, USA.

Several composite oligonucleotides with a 2',5'-linked oligo-adenylate activator of RNase L covalently linked to deoxy-ribonucleotides complementary (antisense) to selected intragenic RSV RNA sequences were evaluated for RSV inhibition. NIH351, the most potent of these antisense chimeras, was directed against highly conserved nucleotides in transcriptional stop/start intergenic sequences within the RSV genome. NIH351 potently inhibited RSV strain A2 in HEp-2-2 cells. The EC_{50} = 0.3 μM by CPE inhibition assay and 0.1 μM by neutral red uptake assay. Using the same assays, ribavirin was active at 40 and 8 μM, respectively. In a virus yield reduction assay, the EC_{90} for NIH351 = 1 μM and for ribavirin = 80 μM. It was similarly active against RSV A2 in embryonic monkey kidney cells. NIH351 also inhibited other RSV strains including a type B virus and a clinical isolate in HEp-2 cells. At 10 μM, the highest concentration tested, the oligonucleotide was not inhibitory to stationary or actively growing cells. Significantly, the scrambled antisense sequence chimera, NIH426, was more than 10-fold less active. The results show that NIH351 is a highly active 2-5A-antisense chimera which is approximately 100 times more potent than ribavirin, the drug of choice for treating RSV infections.

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PCR amplification and determination of the RNA sequences for the P3 coding region of Human Rhinoviral serotypes.

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There are more than 100 serotypes of human rhinovirus (HRV). The genomic sequences for five serotypes are known, as is the sequence of the P3 coding region for one additional serotype. We have designed small molecule inhibitors of the HRV 3C protease which have broad-spectrum activity against rhinovirus serotypes. Therefore, we wished to determine the sequences for 3C protease from as many additional serotypes as possible. The known 3C protease sequences are only about 50% conserved, yet PCR primers were designed which successfully amplify approximately 2kb of cDNA spanning from the middle of 2C to the middle of 3D. The cDNA was synthesized using purified total cytoplasmic RNA from HRV infected HeLa cells. The primers have successfully amplified cDNA from all five serotypes tested to date. The newly determined sequences are highly homologous to known 3C protease sequences but display unique differences amongst themselves, confirming that the PCR primers amplify legitimate HRV viral sequences. Regions of conserved and variable sequences are interpreted in light of our solved structures of inhibitors bound to 3C protease.

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Recombinant human interferon-alpha hybrid B/D protects mice against lethal Ebola virus infection.

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Ebola Zaire virus causes severe hemorrhagic fever in humans, with high mortality rates. There is no effective therapy. Potential antiviral drugs are currently being evaluated by using a mouse-adapted strain of Ebola Zaire virus which is uniformly lethal for adult, immunocompetent mice. Recombinant human interferon-alpha hybrid B/D, which has cross-species antiviral activity in other murine models of viral disease, protected BALB/c mice against lethal Ebola infection. Groups of 10 mice were treated for 6 days with interferon-alpha B/D, beginning on the day of infection, or on day 1 or day 2 postinfection, with a daily dose of 5×10^7 U/kg of interferon. All treated animals remained healthy, while all control mice died. No toxicity was observed. However, this dose of interferon failed to protect mice when started on day 3 postinfection, a point at which mice begin to show signs of illness, and Ebola viral titers in the liver and spleen exceed 10^7 PFU/g. A dose of 5×10^6 U/kg was also fully protective on days 0 or 1, completely to partially effective on day 2, and had no effect on day 3. A dose of 5×10^5 U/kg was fully effective on days 0 and 1, partially effective on day 2, and ineffective on day 3. Combination treatment with interferon-alpha B/D and high-titer anti-Ebola neutralizing antibody is now being studied. Murine interferon-alpha was also partially protective, given in a daily dose of 10^5 U/kg beginning the day before viral challenge. Interferon-alpha B/D also inhibits Ebola virus replication in monkey kidney cell lines. The relative inhibitory effect of alpha-interferons on Ebola replication in murine and primate cell lines is currently under investigation.

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Cidofovir (HPMPC) Treatment of Monkeypox J. W. Huggins*, D. Smee, M. J. Martinez, and M. Bray, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD 21702-5011

Monkeypox virus, which is transmitted to humans by direct contact with infected animals or by exposure to infected individuals, has recently re-emerged in the Democratic Republic of the Congo (DRC). Experimental monkeypox infection of cynomolgus monkeys by aerosol results in significant fever, exanthema, enanthema, and a pulmonary distress syndrome that resembles the course of clinical disease in humans. The viral DNA polymerase of monkeypox, cowpox and variola share significant sequence homology with herpes simplex at the proposed drug-binding site. Screening of DNA polymerase inhibitors identified cidofovir (HPMPC, Vistide™) as the most active in a plaque-reduction assay. Cidofovir was also active in a lethal surrogate mouse model using cowpox virus. Intranasal inoculation of mice with 10^6 PFU of cowpox resulted in weight loss beginning on day 4 and a mean time to death of 8 days. Viral titers reached 10^6 -10^7 PFU/g in the lungs by day 4, then declined. Decreased blood oxygen correlated with severe pulmonary distress observed 1-2 days before death. A single treatment with 100 mg/kg of cidofovir was protective when begun as late as day 5 post infection, and protected 20% of mice when treatment was began on day 6 even though mean time to death was 8-9 days in controls. The ability to successfully treat with cidofovir up to 5 days after infection represents significant antiviral protection. Initial cidofovir trials in cynomolgus monkeys, using true small particle aerosol infection with monkeypox, where treatment was initiated on the day of infection, completely protected the animals from clinical and laboratory signs of disease, while the placebo-treated monkey developed classical poxvirus lesions and pulmonary distress. These results strongly support conducting a controlled clinical trial of cidofovir therapy of monkeypox in the DRC.

Poster Session II: Herpesvirus, Respiratory Viruses, and Other Viral Infections

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BIOLOGICAL ACTIVITIES OF 9-(2-PHOSPHONYLMETHOXY-ETHYL)- N^6-CYCLOPROPYL-2,6-DIAMINOPURINE.

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The antiviral and cytostatic properties of 9-(2-phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine (CPr-PMEDAP) were evaluated in several *in vitro* and *in vivo* test systems. CPr-PMEDAP was shown to be a broad-spectrum antiviral agent with potent activity against human herpesviruses (i.e., HSV-1, HSV-2, VZV and CMV), and HIV. Overall, the antiviral EC_{50} value of CPr-PMEDAP was intermediate between that of 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and its guanine counterpart PMEG. However, the concentration causing inhibition of cell growth (IC_{50}) was markedly lower for CPr-PMEDAP and PMEG than for PMEDAP. In human lymphoid cells (MOLT-4, CEM and Raji) and human erythroleukemia cells (K562) the IC_{50} for CPr-PMEDAP and PMEG was invariably in the same order of magnitude (range: 0.8–2 μM), whereas PMEDAP was approximately 8- to 10-fold less cytostatic. Similarly, CPr-PMEDAP and PMEG had a comparable IC_{50} value in human papillomavirus (HPV)-positive epithelial cells (HeLa and SiHa); PMEDAP was >50-fold less cytostatic. Also, CPr-PMEDAP and PMEG were equipotent in inducing cell differentation in K562 cells. Combination of CPr-PMEDAP with the AMP deaminase inhibitor 2'-deoxycoformycin (dCF) resulted in a 15- to >100-fold increase in IC_{50}. In contrast, the 50% effective concentration (EC_{50}) of CPr-PMEDAP for HSV-1 was decreased by 60-fold upon combination with the IMP dehydrogenase inhibitor mycophenolic acid. Thus it appears that CPr-PMEDAP rather acts as a prodrug of PMEG than of PMEDAP. In a rat choriocarcinoma tumor model, CPr-PMEDAP caused a dose-dependent inhibition of tumor growth; its antitumoral potency was >5-fold higher than that of PMEDAP and 10-fold lower than that of PMEG. Further *in vivo* studies are required to verify whether CPr-PMEDAP may be advantageous to PMEG in terms of antiviral or antitumoral selectivity.

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Anti-herpes Activities of 2-Thio-pyrimidine Nucleoside Analogs; Structure and Activity Relationship.

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Twenty seven 2-thio-pyrimidine nucleosides which have deoxyribose or arabinose in sugar moieties were assessed their antiviral activities against HSV-1, HSV-2, VZV, CMV and TH^-HSV-1. All 2-thiodeoxyuridines with or without 5-halogen bases (F, Cl, Br, I) and 2-thio-5-halogenated deoxycytosines (Br, I) were found to inhibit HSV-1 and HSV-2 whereas did not inhibit TK^-HSV-1 and CMV replications. 2-Thiouracil arabinosides halogenated by Cl or Br. showed also anti-HSV-1 and 2 activities but did not inhibit TK^-HSV-1 and CMV replications. In contrast, 2-thiocytosine arbinosides woth 5-Br or I bases were broadly inhibitory against HSV-1, HSV-2, VZV, CMV and TK^-HSV-1 whereas 5-F or Cl and non-halogenated congeners did not inhibit the replications of any herepsviruses. Among the 2-thio-pyrimidine nucleoside compounds examined, deoxythymidine (TN-17), 5-Cl-deoxycytosine (TN-47), 5-Icytosine arabinoside (TN-32) proved to have the most potent anit-HSV-1, anti-VZV, and anti-CMV activities respectively. The analysis for the mechanism of antiviral activity of these compounds is now in progress.

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CHEMISTRY AND PROPERTIES OF *CYCLO*SALIGENYL-NUCLEOTIDES (*CYCLO*SAL-NMP) OF ACYCLIC NUCLEOSIDE ANALOGUES AS POTENTIAL ANTI-HSV AGENTS

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Different *cyclo*Sal- and α-hydroxyphosphonate pro-nucleotides of the antivirally active acyclic nucleoside analogues acyclovir and penciclovir were prepared and studied concerning their ability to deliver selectively the corresponding nucleotides by chemical hydrolysis. The studies were carried out in phosphate buffer at pH 7.3 and in RPMI culture medium containing 10% heat-inactivated fetal calf serum. As expected, all *cyclo*Sal-phosphotriesters liberated the nucleotides selectively following the previously reported tandem-reaction. Although, the rates of hydrolysis were markedly higher for the acyclovir-derivatives as compared to the penciclovir compounds. This could be due to the additional hydroxymethylene group in penciclovir. In order to prove that assumption blocked *cyclo*Sal-penciclovir monophosphates were prepared and studied. In contrast, the α-hydroxyphosphonate derivatives yielded only the nucleosides or the H-phosphonate monoesters. Consequently, only the *cyclo*Sal-phosphotriesters can act principally as pro-nucleotides in biological systems. Additionally, the partition coefficients of the new compounds in octanol/water were determined in by HPLC-analysis and were found to be considerably higher as compared to the parent nucleosides. Some of the new compounds exhibited significant *in-vitro*-antiviral activity against HSV-1/Kupka and TK-deficient HSV-1 in Vero cells without enhancing the cytotoxicity of the parent nucleoside.

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Mechanism of anti-herpesvirus activity of natural carrageenans of diverse structural types. E.B. Damonte*, M.J. Carlucci*, M. Ciancia, M.C. Matulewicz**, A.S. Cerezo**. *Laboratorio de Virología, Dpto. de Química Biológica and **Dpto. de Química Orgánica. Facultad de Ciencias Exactas y Naturales. Universidad de Buenos Aires. 1428 Buenos Aires. Argentina.**

The antiherpetic activity and mode of action of a variety of natural carrageenans isolated from the red seaweed *Gigartina skottsbergii* were analyzed. The λ-carrageenan $1T_1$, the μ/ν carrageenan $1C_3$ and the κ/ι type $1C_1$ were potent inhibitors of a wide spectrum of strains and clinical isolates of herpes simplex virus (HSV) types 1 and 2, with IC_{50} values ranging from 0.2 to 2.8 μg/ml, as determined by plaque reduction and virus yield inhibition assays. The cytotoxic concentrations (CC_{50}) determined by trypan blue exclusion assay on stationary and proliferative cells, MTT method and radiolabeled macromolecular precursor uptake assay ranged from 500 to >1000 μg/ml, indicating high selectivity indices for these compounds. Time of addition studies suggested that virus adsorption was the main target for antiviral action of the three carrageenans, but $1T_1$ was still significantly inhibitory when added after adsorption. The kinetics of virus binding was highly reduced in the presence of the compounds confirming their action on the first stage of the virus cycle whereas viral internalization and protein synthesis were not affected. The pretreatment of HSV with the carrageenans showed that $1C_1$ and $1C_3$ lacked direct inactivating effect at concentrations near the IC_{50} but 1T1 exerted a strong virucidal action. The cyclization of the α-D-galactose 6-sulfate and 2,6-disulfate units of $1T_1$ to afford the derivative $1T_1T_1$ maintained the antiviral activity in the same level of IC_{50} but totally eliminated the virucidal properties. Thus, the structure of $1T_1$ seems to be responsible of its differential action respect to $1C_1$ and $1C_3$ allowing a more stable binding of the first carrageenan with HSV and leading to virion inactivation. In contrast, $1C_1$ and $1C_3$ fail to bind with high affinity to virus alone but are able to interfere with the interaction between HSV particles and the cell.

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Antiviral Activity of New Piperazinylpyrimidine Derivates

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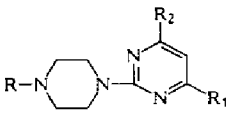
A series of novel piperazinylpyrimidine was sythesized via condensation of N^4-substituted pyperazinyl-(1)-amidine with β-diketone or ester of β-ketoacids and tested for activity against vaccinia virus, herpes viruses and NDV. Some of them were found to inhibit the replication of vaccinia virus, pseudorabies virus in CEF cell system, and herpes simplex virus, in MDBK cells. No activity was found against NDV. PP-105 and PP-103 exhibit antiviral activity against vaccinia virus and herpes viruses with comparable effect at concentration of 1 - 50 μg/ml without being toxic to the host cells in the antiviral dose-range. The both compounds reduced virus infectivity in vitro > 99.9%. The cytotoxicity is low (TC_{50} > 1mg/ml PP-103). The title compounds seems to be a promising candidates for further investigation.



R = alkyl or aryl alkyl
$R_1 = CH_3$
$R_2 = CH_3$, OH

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Synthesis and Antiviral Activity of 6-Chloropurine Arabinosides

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6-Chloropurine arabinoside (**1a**) was obtained by treatment of the 2'-*O*-acetylated congener with ammonia in methanol. The 3',5'-di-*O*-tritylated riboside was allowed to react with diethylaminosulfur trifluoride (DAST) to give the 2'-deoxy-2'-fluoroarabinoside, from which 6-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-purine (**1b**) was obtained. The antiviral effects of **1a,b** were assayed against several DNA and RNA viruses. Compound **1a** displayed potent activity against varicella-zoster virus (VZV) and proved more than 100-fold less active against TK^- VZV than TK^+ VZV, suggesting that its anti-VZV activity at least partially depends on phosphorylation by the VZV-induced thymidine kinase (TK). Compound **1a** showed moderate activity against other DNA viruses, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), and vaccinia virus. It was equally active against TK^- and TK^+ strains of HSV-1, which suggests that the HSV-1-encoded thymidine kinase does not play a role in the anti-HSV-1 activity. No activity was noted with any of the compounds against various RNA viruses at subtoxic concentrations. Compound **1b** also showed some activity against HSV and VZV.

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A New Prodrug Concept for Nucleoside Analogs

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Several nucleoside analogs with potent antiviral or other antimetabolic activities have poor or limited oral bioavailabilities. In the search for enhanced oral uptake of the potent anti-varicella zoster virus (VZV) compound R (-) -9- [4- hydroxy-2- (hydroxymethyl) butyl] guanine (H2G), we discovered the unique properties of the mixed stearyl/valyl di ester. Using rat as a model the oral bioavailabilty was considerably better than for the mono-or bis valyl esters. The mono-or bis-stearyl esters showed very low or no bioavailability. This prodrug of H2G is now in clinical development as an anti-VZV drug (ABT-606). In extended studies, similar prodrugs of other nucleoside analogs with low oral bioavailabilities have been prepared and investigated. Results from these studies will be presented and discussed.

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Development of New Prodrugs of (-)-9-[4-Hydroxy-2-(hydroxymethyl)butyl]guanine (H2G).

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(-) -9- [4 -Hydroxy-2- (hydroxymethyl) butyl] guanine (H2G) is an acyclic nucleoside analog with broad-spectrum antiherpes virus activity. H2G is 20 times more potent than acyclovir against varicella zoster virus in cell culture. The oral bioavailability in rats is 8 % and a systematic study of H2G prodrugs was therefore undertaken to optimize the bioavailability. Screening studies in rats compared the urine recovery of the parent compound (H2G) following an oral dose of the prodrugs to that obtained from intravenous dosing of H2G. More than 100 prodrugs with widely differing structures were synthesized and evaluated, such as carbonates, carbamates and alkyl, aryl, heteroaromatic, sulfonic acid, amino acid, dipeptide and tripeptide esters as well as combinations of these. Most of these gave slightly increased or decreased oral bioavailability compared to oral H2G. A mixed diester of H2G with an amino acid, such as L-valine or L-isoleucine and a long chain fatty acid gave a higher oral bioavailability than any monoester or other diester, including the divalyl ester. The optimal prodrug (R)-9-[2-stearoyloxymethyl-4-(L-valyloxy)butyl]guanine (ABT-606), is now in clinical development for the treatment of shingles.

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INHIBITION OF THE GROWTH OF HERPES SIMPLEX VIRUS TYPE 2 BY VACCINIA VIRIONS.

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Infection of B-SC-1 cells with vaccinia virus following their infection with herpes simplex virus type 2 (HSV-2), results in inhibition of the growth of HSV-2. This inhibition is exerted by extracellular enveloped virus (EEV), as well as by intracellular mature vaccinia virus (IMV), which lacks the additional outer virus membrane found in EEV. Ultraviolet irradiation of vaccinia virus, which efficiently inactivates the infectivity of the virus, has only a slight affect on its capability to inhibit the growth of HSV-2. However, treatment of the purified virus with the detergent Brij-58 or n-octyl-α-D-glucopyranoside abolishes its ability to inhibit HSV-2. A similar result to that observed with the detergent-treated virus is obtained, when purified cores of vaccinia virus are used.

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Inhibitory effect of *cyclo*Saligenyl-Nucleoside Monophosphates (*cyclo*Sal-NMP) of Acyclic Nucleoside Analogues on HSV-1 and EBV

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The antiviral activity of a new series of *cyclo*Sal- and α-hydroxyphosphonate pro-nucleotides of acyclovir and penciclovir against herpes simplex virus type 1 (HSV-1), thymidine kinase-deficient (TK$^-$) HSV-1, and Epstein-Barr Virus (EBV) was evaluated. Using the XTT-based tetrazolium reduction assay EZ4U the derivatives were examined for their antiviral and cytotoxic activites in HSV-1 as well as HSV-1-TK$^-$-infected Vero cells. The anti-EBV activity was assessed by means of an EBV DNA hybridization assay using a digoxigenine-labelled probe specific for the Bam H!-W-fragment of the EBV genome and by measuring viral capsid antigen (VCA) expression by immunofluorescence after a 7-day incubation period of P3HR-1 producer cells with the test compounds. Some of the new synthesized pro-nucleotides proved to be potent and selective inhibitors of HSV-1 replication, EBV DNA synthesis, and EBV VCA expression.

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INTERFERENCE OF ZINC WITH PENTOSAN POLYSULFATE IN THE HSV-1/VERO CELL SYSTEM

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The topical application of zinc and heparin containing gels in the therapy of cutaneous herpes simplex virus infections prompted us to study the in vitro effect of the heparinoid pentosan polysulfate (PPS) and of zinc sulfate in Vero cells infected or not with herpes simplex virus type 1 (HSV-1). The antiviral activity was assessed with morphological and biochemical criteria (MTT method). PPS was found to inhibit virus replication during and after virus adsorption and expressed an additional antiviral effect, if applied to cells before virus infection. In contrast herewith, zinc sulfate did not show any antiviral activity in the HSV-1/Vero cell system and consequently, it was unable to support the anti-HSV-1 effect of PPS. Although the well-known cell-protecting activity of very low zinc sulfate concentrations could be confirmed, higher concentrations showed a deleterious cytotoxic effect and interfered significantly with the antiviral activity of PPS. Similar results were found with zinc and the polyanionic oxidation product of caffeic acid (KOP). Concluding from the results, a very carefully estimation of the risc-benefit ratio of zinc-containing anti-HSV drugs seems to be necessary.

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Application of Antiviral and Local Anaesthetics or Anti-inflammatory Agents for the Topical Treatment of Herpes Simplex Virus Assessed Using a Murine Zosteriform Infection Model.

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We have reported the modification of the zosteriform murine infection model which employs the adoptive transfer of immune cells (ATI) to recipient infected mice to produce a disease that mimics human recurrent HSV. Mice were infected with HSV-1 by scarification at the lateroventral line of the neck; two days later, the mice received immune cells from HSV-1-infected syngeneic mice. Although virus was cleared more quickly from the target tissues of virus replication in recipient mice, ATI resulted in a heightened inflammatory response, and delayed healing. This model was used to test the effects of topical foscarnet (3%) TID followed two hours later by the application of local anaesthetics or anti-inflammatory agents TID. Virus clearance and clinical signs, including ear thickness and zosteriform spread of lesions, were studied. Treatment with 3% foscarnet followed by placebo cream accelerated virus clearance but had little effect on clinical parameters. Treatment of foscarnet followed by Xylocaine (lidocaine 5%), or EMLA (lidocaine 2.5% and prilocaine 2.5%) enhanced virus clearance but significantly increased the clinical signs, ear thickness, and zoster score. Treatment with foscarnet followed by Oruvail (ketoprofen 2.5%) prolonged virus replication for two days and significantly increased clinical signs. Treatment of foscarnet followed by Amuno gel (indometacin 1%) or Voltaren Emulgel (diclofenac 1%) led to 100% mortality of mice after few days of application. In contrast, foscarnet followed by Preferid (budesonide 0.025%) or Hydrocortisone (hydrocortisone 1%) produced a marked reduction in clinical signs, ear thickness and zoster score although virus replication was prolonged for two days. These results are discussed in relation to the inflammation and discomfort experienced by patients and a possible role for anti-inflammatory, anaesthetics and antiviral formulations in the treatment of HSV reactivation episodes in man.

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The Effects of a Topical Serine-protease (PHM-101) on HSV-1 Pathogenesis Assessed in a Murine Neck-ear Zosteriform Infection Model for the Disease.

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PHM-101 is a natural product and a proteolytic enzyme of the serine-protease variety. The compound is believed to have an anti-inflammatory and antiviral activity and in this study comparisons were made with commercially available antiherpes cream containing acyclovir 5%. Mice were infected with HSV-1 by scarification at the lateroventral line of the neck and lesions started to appear at the ipsilateral ear pinna on day 5 or 6 p.i. Mice were treated topically on the ear b.i.d. for the period of 3-9 or 5-9 days and a group of mice was left untreated. Clinical signs were recorded and levels of infectious virus in the target organs were determined. The pathogenesis of infection followed the predicted pattern in the untreated control mice. All formulations were tested blind, well-tolerated by all the animals and no apparent itching, pain, scratching or redness was seen. When the results were decoded, the formulation which contained purified PHM-101 was shown to be superior in terms of clinical response and antiviral activity compared to a partially purified preparation containing PHM-101 at a lower concentration. Furthermore both the test compounds compared favourably in terms of clinical response to 5% acyclovir cream.

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A HSV helicase-primase inhibitor, BILS 45 BS, with potent oral activity against acyclovir-resistant infection in nude mice

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The present study reports on the activity of BILS 45 BS against acyclovir-resistant (ACV^r) HSV-1 infections in nude mice. BILS 45 BS is a selective HSV helicase-primase inhibitor that inhibits the helicase, primase and DNA-dependent ATPase activities of the enzyme with an IC_{50} of 0.9, 0.3 and 0.11 µM, respectively. *In vitro*, BILS 45 BS was more potent than ACV against wild-type HSV-1 and HSV-2 strains, and ACV^r HSV isolates, as determined by a standard plaque reduction assay with EC_{50} values of about 0.15 µM. *In vivo*, BILS 45 BS exhibited oral activity against both HSV-1 and HSV-2 infections. In this study, we evaluated the antiviral activity of BILS 45 BS against ACV^r infections mediated by HSV-1 *dl*sptk and PAA^r5 mutants which contain mutations in the viral thymidine kinase and polymerase genes, respectively. Following cutaneous infection of nude mice, both HSV-1 *dl*sptk and PAA^r5 induced significant, reproducible and persistent cutaneous lesions that lasted for more than two weeks. Oral treatment with acyclovir (100 or 125 mg/kg/day, tid by gavage) did not affect either mutant-induced infections. In contrast, BILS 45 BS at a dose of 100 mg/kg/day almost abolished cutaneous lesions mediated by both ACV^r HSV-1 mutants. The ED_{50} of BILS 45 BS was 55 and 63 mg/kg/day against *dl*sptk and PAA^r5-induced infections. Oral bioavailability in mice was 49% with a C_{max} of 31.5 µM at a single dose of 25 mg/kg. Our results, for the first time, demonstrate a very effective oral therapy of ACV^r HSV-1 infections using a selective HSV helicase-primase inhibitor.

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Mycophenolate Mofetil Ointment Potentiates the Activity of Systemically Administered Acyclovir in Murine Models for Cutaneous Herpesvirus Infection.

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Mycophenolate Mofetil (MMF) is a novel immunosuppressive molecule that is used in transplant recipients. These patients may develop opportunistic herpesvirus infections for which antiviral therapy is needed. Thus, MMF may be used concomitantly with acyclovir (ACV) for the treatment of such infections. We previously demonstrated that MMF markedly potentiates the anti-herpesvirus activity of acyclovir, ganciclovir and penciclovir. We have now extended this study and shown that topically applied MMF potentiates the anti-herpes virus activity of systemically administered acyclovir. In a first set of experiments hairless mice were infected at the lumbrosacral area with HSV-1 (KOS) and were either treated for 5 consecutive days with (i) 20 mg/kg/day of ACV *via* subcutaneous (sc) injection or (ii) 5% MMF ointment or (iii) combination ACV (sc) and 5% MMF ointment. Neither ACV alone nor MMF alone conferred protection against virus-induced mortality. However, when mice were given ACV systemically together with MMF ointment, 80% remained disease-free. In a second set of experiments, athymic nude mice were infected at the lumbrosacral area with an ACV-resistant HSV-2 strain that was isolated from a patient with an ACV-refractory mucocutaneous lesion. Animals were either treated for 8 consecutive days with either (i) ACV at 100 mg/kg/day *via* sc injection, or (ii) 5% MMF ointment, or (iii) the combination ACV (sc) and 5% MMF ointment. Neither ACV alone nor MMF alone protected the mice against the infection, whereas 60% of the animals that received the combined treatment remained disease-free. Thus, topical administration of MMF in patients with an ACV-resistant (muco)cutaneous herpesvirus infection may make systemically administered ACV effective in the treatment of these infections.

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Denavir, Zovirax Cream and Zovirax Ointment in the Topical Treatment of Experimental Dorsal Cutaneous Herpes Simplex Virus Type 1 (HSV-1) Infection in the Guinea Pig. M. McKeough and S. Spruance, U. of Utah, Salt Lake City, UT, USA.

Denavir([DEN], 1% penciclovir cream, SmithKline Beecham, Pittsburgh, PA) and Zovirax cream([ZC], 5% acyclovir cream, GlaxoWellcome, RTP, NC) are approved for the episodic treatment of herpes labialis in immunocompetent persons in many countries. We compared the two directly at a dosing regimen of 1x/day for three days in our guinea pig model (irritation precluded more frequent dosing). Briefly, we infected four sites on the backs of female Hartley outbred guinea pigs with HSV-1 by multiple punctures with a vaccination device (Day 0) and evaluated the efficacy on Day 4 at each infection site by measurements of lesion number, area and virus titer. DEN compared to ZC achieved modest but statistically significant better reductions in lesion number and total lesion area. Reductions in lesion virus titer were similar. Because differences in the dermal irritation caused by the drug vehicles in this model might have influenced measurements of lesion number and area, we repeated the study and compared each drug to its respective vehicle in the model and included Zovirax Ointment([ZO],5% acyclovir ointment, GlaxoWellcome) as a control.

	Experiment 1		Experiment 2					
Measure	DEN	ZC	DEN	CRM	ZC	CRM	ZO	OINT
# of lesions	50*	55	41*	49	52	52	49	48
Area (mm^2)	202*	265	151*	231	185*	295	170*	235
Virus titer	4.42	4.38	4.08*	4.99	4.40*	5.10	4.27*	4.87

(Data are median values; *, $p<.05$; n = 9 to 16; titer is $\log_{10}$pfu/ml)

For the drug/vehicle experiment, DEN worked significantly better than ZC in reducing virus titer ($p = .04$) and significantly better than ZO by all three measures ($p = .02, .03, .01$). ZC and ZO were not significantly different by any measure. These data indicate a relative potency of DEN > ZC = ZO in this model. The relative potency of these formulations against herpes labialis should be compared in clinical trials.

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Famciclovir treatment of acute herpes simplex virus infection in mice reduces the subsequent frequency of latent virus reactivation induced by transitory hyperthermia.

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Thackray and Field (J. Infect. Dis. 173: 291-299, 1996) have previously reported that treatment of acute cutaneous herpes simplex virus (HSV) infection of mice with famciclovir (FCV), but not valaciclovir (VACV), markedly reduced the subsequent ability to reactivate latent virus in explanted sensory ganglia. The activity of FCV appears to be due to a quantitative reduction in the number of latently infected neurons within ganglia (Thackray and Field, Antiviral Res. 30: A15, 1996, Field et al, Abstracts of the 37th ICAAC, H43, 1997). In order to determine the consequence of this effect in the whole animal, transitory hyperthermia was used to induce virus reactivation in latently infected mice. Mice were inoculated in the ear pinna with HSV-1 SC16. FCV or VACV was administered at 1 mg/ml (approximately 200 mg/kg per day) via the drinking water from day 2 to 10 post infection (pi). Both compounds were equally effective at reducing the severity and healing time of the primary infection relative to placebo. Lesion AUC values for placebo, FCV and VACV treated animals were 29.2, 9.7 and 5.6 respectively. Two to three months pi, animals were exposed to transitory hyperthermia, and trigeminal ganglia subsequently examined for infectious virus. No reactivation was observed without hyperthermia stress. Following hyperthermia, reactivation was detected in significantly fewer ($P<0.05$) FCV-treated mice (4/17; 24%) compared with placebo (13/18; 72%) or VACV-treated animals (12/19; 63%). These data show that early FCV- (but not VACV-) treatment of primary HSV infection can reduce the subsequent incidence of virus reactivation in mice, and suggest that the observations of Thackray and Field have significance beyond the latency model that they used.

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Antiviral activity of RD6-2198 against intravaginal infection with herpes simplex virus type 2 in mice.

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We have previously shown that RD6-2198 had potent antiviral activity against several enveloped viruses in cell culture (Fujiwara et al.; 10th ICAR). In these viruses, human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus (HSV) of sexually transmissible virus were included. In expectation of drug for sexually transmissible virus, we evaluated the antiviral effect of RD6-2198 using intravaginal infection with HSV-2 in mice, because there is no small animal model to study intravaginal HIV infection. When RD6-2198 (10 mg/ml) was applied at 30 min before intravaginal infection, no antiviral effect was detected. When the intravaginal infection was carried out in the presence of RD6-2198 at a concentration of 10 mg/ml, the development of lesions and virus-induced death was protected completely. In contrast, dextran sulfate (DS), using as a control, showed no complete protection at a concentrations of 300 mg/ml, though the survival rate was prolonged at this concentration. Virus titers from vaginal swabs were also determined at 3, 5 and 7 days post-infection. No virus was recovered from RD6-2198-treated mice. Virus titers of 300 mg/ml DS-treated mice that had lesions were lower than those of control mice. Virus titer did not decrease in DS-treated mice at a concentration of 100 mg/ml or below. In conclusion, RD6-2198 should be considered as a promising candidate for the prevention of sexually transmission of HIV and HSV.

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Efficacy of Topical Acyclovir Monophosphate in Genital HSV-2 Infections of Guinea Pigs. J. Palmer[1], E. Harden[1], G. Szczech[2], G. Painter[2], S. McLean[3], K.Y. Hostetler[4], and E. R. Kern[1]. [1]Univ of Alabama Sch of Med, Birmingham, AL; [2]Triangle Pharmaceuticals, Inc., Durham, NC; [3]Lipoderm, Inc., Halifax, Nova Scotia; and [4]Univ of California, San Diego, CA.

The purpose of these studies was to compare the efficacy of Acyclovir (ACV) and ACV Monophosphate (ACVMP) against ACV sensitive and resistant genital HSV-2 infections of guinea pigs. Groups of 10 female Hartley guinea pigs were infected intravaginally with $2x10^4$ pfu HSV-2 and treated with PBS, placebo, 5% ACV in PEG, or 5% ACVMP in PEG or liposomes beginning 24h post-infection and continued 3 times/day for 7 days. Lesions were graded daily and viral titers from vaginal swabs and external genital lesions were determined. ACVMP in PEG vehicle at pH 8.0 or at pH 5.5 gave significant decreases in both vaginal and lesion virus titers against HSV-2 strain G. Lesion scores were also significantly decreased. A significant, but inconsistent, effect with ACV in PEG was observed for some of the parameters. The formulation of 5% ACVMP in liposomes also significantly decreased virus titer-day and lesion titer-day values for both G and an acyclovir resistant strain, 12247. Highly significant decreases in viral titers and lesion scores were observed in other guinea pigs given a single treatment of ACVMP in PEG or liposomes 2 hrs before viral inoculation with either strain G or with another ACV-resistant strain AG-3. These results indicate that topical ACVMP is at least as effective as ACV in PEG and that efficacy can be improved through the use of alternative vehicles.

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In Vitro and *In Vivo* Evaluation of the Activity of Stannous Compounds Against Herpes Simplex Virus.

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Some stannous (tin-containing) compounds have broad antimicrobial activity and have found commercial use in oral hygiene products. Because the antiviral potential of stannous has not been explored we investigated the anti-HSV activity of two compounds, stannous fluoride (SnF2) and stannous gluconate (SnGluconate). Dilution's of both compounds (0.05%-0.5%) were formulated in 50% glycerol:50% H_20 and tested in plaque reduction assays using HeLa cells for activity against HSV-1 and HSV-2. SnF2 was tested against HSV-2 strains MS, 333 and an acyclovir resistant HSV strain 333 TK-. The 50% effective dose (ED_{50}) of 0.11%, 0.13% and 0.15% respectively for these strains were observed. HSV-1 strains 17syn+ and KOS exhibited ED_{50}'s of 0.135% and 0.15%. SnGluconate tested against HSV-2 MS strain resulted in an ED_{50} of 0.285%. *In vivo* studies were conducted using the guinea pig model of recurrent genital herpes. Animals were treated with SnF2 Gel-Kam (0.4%)and SnGluconate (1.5%) 3 times daily beginning on day 21 after intravaginal inoculation with $5x\ 10^5$ pfu's of HSV-2 strain MS. Animals were monitored for 48 days to determine frequency and severity of infection. Treatment with SnF2 or SnGluconate resulted in reduction of recurrences compared to untreated controls. These results are encouraging and suggest that stannous compounds might be useful in the treatment or prevention of HSV infections. Further studies are needed to determine the mechanism of action of this unique class of anti-herpes drugs.

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Evaluation of Triclosan as a Possible Intravaginal Microbicide:*In Vitro* and *In Vivo* Activity Against Herpes Simplex Virus Type 2 (HSV-2)

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The development of safe and effective intravaginal microbicides is one strategy for reducing the transmission of genital herpes. Nonionic surfactants are logical candidates for development as microbicides because they have broad anti-bacterial and virucidal activities and some, such as Triclosan (2,4,4,'-trichloro-2'-hydroxydiphenyl ether) have favorable safety profiles. In evaluating Triclosan we determined by plaque reduction assay using HeLa cells and HSV-2 strain MS strain that the concentration required to inhibit 50% of virus was 0.18mg/ml. *In vivo* effectiveness was assessed using Swiss Webster mice primed with medroxyprogesterone and inoculated intravaginally with 4 log10 PFU HSV-2 strain 186. With this model <10% of unprotected (control) mice survive the infection. Varying doses of Triclosan were formulated in glycerol and PBS and intravaginally administered to mice immediately prior to HSV-2 challenge resulting in 93% survival with 10 mg/ml and 100% survival with 50 and 100 mg/ml. Triclosan at a concentration of 100 mg/ml formulated in glycerol and PBS was ineffective when treatment was given 5 minutes before challenge (10% survival) but the same formulation with standard agar (10 mg/ml) provided substantial protection (73%). These very encouraging results demonstrate that Triclosan, a broadly active antimicrobial agent, is active *in vitro* and *in vivo* against HSV-2 and should be considered a candidate for clinical evaluation as an intravaginal microbicide.

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Evaluation of Undecylenic acid as a topical microbicide against genital herpes infection in mice. N. Bourne, J. Ireland, and DI Bernstein. Children's Hospital Research Foundation, Cincinnati OH.

There is increasing interest in the use of topical microbicides to help prevent the spread of sexually transmitted diseases. Undecylenic acid (UA), a monosaturated fatty acid, is the active ingredient in a number of over the counter antifungal spray powders but also has antibacterial and antiviral activity. UA has been shown to have in vitro activity against HSV and has been evaluated as a treatment in animal models and humans. We therefore evaluated UA as a topical microbicide against genital HSV infection. Swiss Webster mice were administered 15 ul of a 20% solution of UA in polyethylene glycol (PEG) vehicle, vehicle alone, or PBS intravaginally immediately prior to challenge with 10^4 pfu HSV-2 strain 186. In 2 studies a total of 28/29 PBS control animals became infected, 25 developed symptomatic genital herpes and 18 died. Pretreatment with PEG vehicle reduced the number of animals that became infected (22/31, $p<0.05$), but not the number of animals that developed symptoms (21) or died (18). In contrast only 3/31 animals that were treated with UA had evidence of viral infection ($p<0.00001$ vs PBS or PEG control), developed symptoms ($p<0.00001$) and died ($p<0.0005$). Thus, UA is an approved OTC medication that provided significant protection against HSV disease and infection making it an excellent microbicide candidate.

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Pattern of Cross-Resistance between Phosphonylmethoxyethyl-guanine (PMEG) and Cyclopropylphosphonylmethoxyethyl-2,6-diaminopurine (CPr-PMEDAP) and Other Anti-Herpesvirus Agents

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The pattern of cross-resistance between PMEG and CPr-PMEDAP and other anti-herpesvirus drugs including phosphonylmethoxyethyl derivatives of adenine (PMEA) and 2,6-diaminopurine (PMEDAP), 3-hydroxy-2-phosphonylmethoxy-propyl derivatives of cytosine (HPMPC) and adenine (HPMPA), acyclovir (ACV), brivudin (BVDU), penciclovir (PCV), ganciclovir (GCV), the pyrophosphate analogues foscarnet (PFA) and phosphonoacetic acid (PAA) have been analyzed using herpes simplex virus type 1 (HSV-1) strains that were selected *in vitro* under pressure of PMEG and CPr-PMEDAP. 50% Inhibitory concentrations (IC_{50}) towards for the wild-type HSV-1 strain KOS were 0.002 ± 0.0007 and 0.093 ± 0.01 µg/ml for PMEG and CPr-PMEDAP, respectively, while the IC_{50} values for PMEA and PMEDAP were 6.3 ± 3.3 and 1.6 µg/ml, respectively. The pattern of drug susceptibility/resistance observed with the PMEG resistant ($PMEG^r$) and the CPr-$PMEDAP^r$ strains was similar to that obtained with the $PMEA^r$, $PMEDAP^r$ and PFA^r strains. Thus, resistance to PMEG and CPr-PMEDAP was accompanied with cross-resistance to PMEA, PMEDAP and PFA. Decreased sensitivity to ACV and PCV was noted, while the sensitivity to GCV, BVDU, HPMPC and HPMPA was not modified. Whether the $PMEG^r$ and CPr-$PMEDAP^r$ strains present the same mutations in the DNA polymerase as the $PMEA^r$, $PMEDAP^r$ and PFA^r strains is currently under investigation.

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Characterization of Herpes Simplex Virus Type 1 (HSV-1) Mutants Arising After Multi-step *Versus* Single Round Selection with Foscarnet (PFA) and the Phosphonylmethoxyethyl (PME) Derivatives of Adenine (PMEA) and 2,6-Diaminopurine (PMEDAP)

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We have already shown that multi-step drug selection of HSV-1 with either foscarnet (PFA) or the PME derivatives of adenine (PMEA) and 2,6-diaminopurine (PMEDAP) resulted in an homogeneous viral population that was resistant to PFA and the PME derivatives: all the $PMEA^r$, $PMEDAP^r$ and PFA^r plaque-purified mutants presented the same Ser to Asn change at position 724 in the viral DNA polymerase. We have now determined the phenotype and genotype of different clones isolated after single-round selection with a high concentration of PMEA, PMEDAP or PFA. Approximately 10% of the clones screened proved to be resistant to the pyrophosphate analogues and the PME derivatives. To determine the presence of the specific mutation at position 724 of the DNA polymerase in the various mutants, sequencing was performed on the viral DNA with specific primers, using sequenase. Interestingly, not all the clones presented the Ser to Asn change at position 724. In order to identify the new mutations that occurred in these clones, the subcloning and the analysis of the nucleotide sequence of the DNA polymerase gene was performed. We could identify three different mutations in two $PMEA^r$ clones: 33 Gly → Ser, 522 Ser → Asp and 959 Arg → His. Other clones were examined for the presence of these specific mutations at positions 33, 522 and 959. Some PFA^r, $PMEA^r$ and $PMEDAP^r$ clones presented only the mutations at positions 33 and 522 but not at position 959. Whether these clones harbor other mutations is currently under investigation.

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Establishment of herpes simplex virus thymidine kinase expressing cell line and characterization of an acyclovir-resistant mutant by using it.

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To understand drug mode of action and to have a system to examine cytotoxicity of TK-dependentely activated compounds, we have constructed a plasmid pFTK1 by inserting DNA fragment containing thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) strain F to eukaryotic expression vector, pcDNA3.1/His A. TK-deficient 143B cells were transfected with it by calcium phosphate-DNA coprecipitation method and neomycin-resistant cells were selected. Firstly, insertion of TK fragment was confirmed by DNA hybridization method and cell survival in the presence of HAT in the medium and TK activity of cellular crude extracts were examined. Viral TK expressing cell line, FTK1/143B1 was established and used for characterization of AR1, one of the laboratory derived acyclovir (ACV)-resistant mutants of HSV-1 strain F. It has shown no TK activity when AR1-infected Vero cell crude extracts were tested with thymindine as a substrate and no symptom when AR1 was infected either percutaneously or intranasally in the mice including the fact of its various antiviral resistance to certain nucleoside analogues. One amino acid change, methionine to lysine at 322 position of ORF of the TK gene was detected. Compared were sensitivity to ACV of AR1 and its wild type F in Vero, 143B and FTK1/143B1 cell culture systems. F showed no significant difference in antiviral sensitivity in all systems, but AR1 lost its resistance to ACV in FTK1/143B1 cells since it showed the resistance in Vero and 143B cells. So it can be concluded drug-resistance of AR1 is caused by TK-deficiency. More data regarding antivrial activity and cytotoxicity of various nucleoside analogues by using FTK1/143B1 cell line will be reported and discussed.

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Phenotypic and genotypic characterisation of clinical isolates of herpes simplex virus resistant to acyclovir.

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A panel of 10 (6 type-1 and 4 type-2) acyclovir resistant thymidine kinase deficient clinical isolates of herpes simplex virus were identified from the UK/European sensitivity monitoring survey carried out between 1980 and 1995. This panel of viruses has now been further characterised. A minimum of 10 double plaque purified clones were prepared for each of the clinical isolates. Sensitivity data on these cloned viruses revealed that 3/10 of the clinical isolates were mixtures containing up to 20% of wild type virus. The pathogenicity of the representative resistant cloned virus was determined using a zosteriform model of herpes virus infection in female Balb/c mice. In this model wild type virus primary infection on the neck results in neuronal spread of virus, the establishment of latency and secondary infection of the pinna of the ipsilateral ear. Laboratory generated thymidine kinase deficient resistant variants show reduced pathogenicity at the peripheral site and are unable to reactivate from ganglia to produce secondary disease. The findings with the clinical isolates investigated to date in this animal model are consistent with virus of a TK deficient phenotype. Sequence analysis of the thymidine kinase gene from the cloned TK deficient viruses used in the animal studies has revealed various nucleotide substitutions or insertions resulting in premature termination of the TK polypeptide. Consistent with published thymidine kinase sequence data, a significant proportion of these insertions occur in the "G-string" motif.

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In vitro susceptiblity to Ganciclovir (GCV) and Foscarnet (PFA) in HIV infected patients: Correlation with therapeutic failure and CMV shedding after treatment.

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We studied the in vitro susceptibility to GCV and PFA in HIV infected patients treated by GCV and/or PFA, with one or more positive CMV isolation after treatment. We compared in vitro resistance to clinical outcome. Seventy five naive patients and 30 GCV and /or PFA treated patients for 1 to 24 months (median treatment = 10 months) were studied. 197 GCV and PFA in vitro tests were performed on 117 strains from untreated patients and 80 strains from treated patients. ID50 and ID 90 were determined by linear regression after plaque reduction assay and compared with ID50 and ID90 of the AD169 strain determined simultaneously. Sensitivity indices SI50 and SI90 were calculated (Pepin et al 1992). Sensitivity criteria to GCV SI50 and SI90 < 3 and to PFA SI50 and SI90 < 4. Medians of ID50 (µM), ID90 (µM), SI50 and SI90 to GCV and PFA are summerized as follows :

		ID50	ID90	SI50	SI90	Sensitive	resistant
Naive strains	GCV	4,5	15	1,2	1,2	n = 117	n = 0
	PFA	48	170	2,2	2,1	n = 117	n = 0
Treated strains	GCV	6,5	23,5	1,7	1,7	n = 54	
	PFA	67,5	200	2,7	2,7	n= 73	
	GCV	**28,5**	**125**	**9**	**9**		n = 26
	PFA	**110**	**580**	**5,5**	**7**		n = 7

GCV and PFA resistance was found in 43% and 24% of treated patients respectively.

We found a correlation between in vitro resistance to GCV or PFA and the CMV clinical progression (92% and 100% respectively). CMV shedding in treated patients was correlated with CMV disease progression (73%). After three months therapy 14/23 (61%) of these patients were resistant to GCV and/or PFA.

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Plaque Autoradiography (PA) in Combination With Plaque Reduction Assay (PRA) for the Evaluation of Herpes Simplex Virus (HSV) Antiviral Resistance in a Clinical Trial. SL Sacks[1,2], P Crosson[1], and BA Rennie[1]. [1]Viridae Clinical Sciences, [2]Univ. of B.C. Dept. of Pharmacology and Therapeutics, Vancouver, B.C., Canada, and the Task Force on HSV Resistance.

Widespread use of thymidine kinase (TK)-dependent antivirals for HSV has led to concern over the possible development of resistance. PRA is the standard method for evaluating resistance, but may lack the sensitivity to detect resistant low proportion viral sub-populations. Our objective was to use PA to evaluate viral isolates (VI) recovered from immunocompetent patients receiving either low dose or full dose oral acyclovir (ACV) for the treatment of a single episode of recurrent genital herpes. First and last VI from 10 patients were examined by PRA and PA in Vero cells. PA was performed by evaluation of approx 1,000 plaques/isolate using TK-dependent uptake of ^{125}IdC in inoculated Vero monolayers followed by X-ray film exposure. The number of PA-plaques was compared to the number of visual-plaques seen in fixed and stained cell monolayers. Relative TK activity was expressed as the percentage of TK-positive plaques. When first and last VI for each patient were compared by PRA the average ACV IC_{50} was 0.37 ± 0.71 and 0.36 ± 0.47 µg/ml, respectively. The TK activity, as measure by PA, was 99.9 ± 6.5% and 100.3 ± 6.4% for first and last VI, respectively. No differences in antiviral susceptibility were seen between first and last VI, and relative TK activity by PA was found to be consistent with PRA susceptibilities with an acceptable background. PA may serve as a valuable tool for increasing the sensitivity of detection of resistant sub-populations of TK-negative HSV.

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Similarities and differences in the genotoxic and apoptosis-inducing capacity of ganciclovir and penciclovir, respectively, in HSVtk$^+$ transfectants of Chinese hamster ovary cells.

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CHO-9 transfectants expressing the thymidine kinase gene of HSV-1 are about 300 times more sensitive against the cytotoxic action of ganciclovir (GCV) and penciclovir (PCV) than their non-transfected counterparts (CHO neo cells). GCV is an extremely potent inducer of sister chromatid exchanges and chromosomal aberrations in HSVtk$^+$ cells. The extent of cytogenetic damage depends on the experimental protocol, i.e. on the length of exposure and recovery time, and appears to rely on GCV incorporation into cellular DNA. On the other hand, at equitoxic concentrations PCV is much less effective in inducing recombinogenic and clastogenic damage in HSVtk$^+$ cells than GCV. As revealed by the terminal deoxynucleotidyl transferase assay and in the CPP32 assay, the major cause of cell death induced by both virostatics is apoptosis. Our findings might be of considerable importance with respect to the gene therapy concept for the treatment of malignant tumors (HSVtk transfection / GCV administration).

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Clinical Trials of Treatment of Meniere's Disease with ACV

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The latent infection of HSV and VZV in trigeminal and facial nerves related to the diseases has been reported and, more recently, latent infection of these viruses in vestibulocochlear nerve has been strongly suggested. I hypothesized the cause of Meniere's disease(MD) is the infection of HSV and/or VZV and administered Acyclovir(ACV) to the patients with MD and reported the marked effectiveness of the treatment at the 9th ICAR. I have accumulated the cases and reconfirmed the results with confidence.

From October 1990 to October1997, I have treated 301 patients with MD administering ACV 2,000mg/day for the average period of two weeks. Out of these 301 cases who visited my clinic suffering from vertigo, ear tingling and impaired hearing, 10 were diagnosed at my clinic and the rest was diagnosed by otolaryngologists. 135 cases were diagnosed as MD, 99 as Meniere's syndrome, 57 as vestibulodysfunction and 15 as other diseases. 96 cases were male (19 - 81 yrs old) and 205 were female(19 - 84 yrs old).

The ratio of effective cases was more than 80 percent, including effective and extremely effective. Among those cases, 39 patients experienced recurrence but the symptoms were much less severe. Upon the patients request, ACV was administered to the recurred cases, and led them to prompt recovery from the disease.

The statistical data of effectiveness in these 301 cases will be shown and some of the typical recovering processes will be introduced.

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In Vitro Activity of Methylenecyclopropane Analogues of Nucleosides Against Cytomegalovirus Infections. C.B. Hartline[1], J. Zemlicka[2], Y-L Qiu[2], D.J. Bidanset[1], E. Harden[1], J.-P. Sommadossi[1], and E. R. Kern[1]. [1]University of Alabama School of Medicine, Birmingham, AL; and [2]Wayne State University School of Medicine, Detroit, MI, USA.

Treatment for cytomegalovirus (CMV) infections, particularly in the immunocompromised host, is not optimal and there is still a need for better therapeutic agents for use in these diseases. We have evaluated a number of both Z and E series of methylenecyclopropane analogues for activity against the AD169 strain of CMV. Eight of these compounds were found to have good activity against HCMV that was comparable to ganciclovir (GCV). Additionally all eight compounds were also active against murine CMV. Two of these compounds (QYL-438, QYL-769) were selected for additional studies. Both compounds were active against a panel of four additional strains of HCMV and were also active against four isolates that were resistant to GCV. In addition to their being active against murine CMV, both compounds also had excellent activity against rat CMV and Rhesus monkey CMV and moderate activity against guinea pig CMV. Both QYL-438 and QYL-769 were nontoxic to human and mouse fibroblast cells. In a clonogenic bone marrow progenitor cell assay using CFU-GM and BFU-E cells, both of these compounds were considerably less toxic than GCV or AZT. These results indicate that some of the methylene-cyclopropane analogues are very active against CMV in tissue culture and at least two of these compounds should be evaluated further in animal model infections as potential candidates for use in treatment of CMV diseases in humans.

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Effective Treatment of Murine Cytomegalovirus Infections with Methylenecyclopropane Analogues of Nucleosides. R.J. Rybak[1], J. Zemlicka[2], Y.-L. Qiu[2], and E.R. Kern[1]. [1]University of Alabama School of Medicine, Birmingham, AL., and [2]Wayne State University School of Medicine, Detroit, MI, USA.

A number of compounds in this class have exhibited good activity against human and murine cytomegaloviruses in tissue culture. The purpose of these studies was to evaluate the efficacy of several of these analogues in murine cytomegalovirus (MCMV) infections in mice. Intraperitoneal (i.p) inoculation of 3-week old Balb/c mice with 2.0×10^5 pfu of MCMV results in an acute, lethal infection with rapid virus replication in visceral and glandular tissue, and an ideal model for identifying compounds that have potential for use in humans. Compounds QYL-284A and QYL-438 were administered i.p. twice daily for 5 days initiated 6, 24, or 48 hours post-infection. Significant protection was demonstrated at 50 mg/kg and 16.7 mg/kg compared to placebo with comparable efficacy to ganciclovir (GCV). When delivered orally once or twice daily at 100 mg/kg/day, both drugs had activity only at the high concentration, but were less effective than GCV. In contrast, another analogue, QYL-769, was found to be highly efficacious when given orally twice daily for 5 days. Mortality of 0% and 13% was observed at 60 mg/kg and 20 mg/kg, respectively, and was similar to GCV. Oral treatment with QYL-769 or GCV reduced virus replication in target organs, but neither resulted in complete clearance of MCMV. Those data indicate that these new analogues have activity comparable to GCV and should be evaluated further to assess their potential for use in humans.

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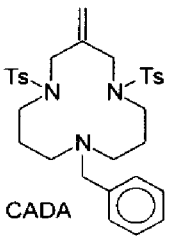
CADA Compounds: Novel Chemotherapeutic Agents for the Human Cytomegalovirus (HCMV). Thomas W. Bell,[1] Qi Jin,[1] Meinrado F. Samala,[1] Albert van Geelen[2] and Stephen C. St. Jeor,[2] Departments of (1)Chemistry and (2)Microbiology, University of Nevada, Reno, Nevada 89557.

Cyclotriazadisulfonamide (CADA) is a synthetic macrocycle that has been found through NIH screening programs to inhibit HIV (EC_{50} 0.1-2 μM), as well as VZV (EC_{50} 3-2 μM). Now a series of nonmacrocyclic (open-chain) and ring-substituted analogs of CADA have been synthesized and screened for activity against herpes viruses. Two new series of anti-HCMV compounds have been identified (EC_{50} 2-5 μM); one is nonmacrocyclic and the other retains the central 12-membered ring of CADA, bearing various groups in place of the benzyl "tail" substituent. 3D QSAR studies have been conducted by means of comparative molecular field analysis (CoMFA). Isotopic labeling, drug distribution and time-of-addition experiments have also been performed as initial probes of the mechanism of action.



CADA

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***In Vitro* Antiviral Effect of 2'-Fluoro-4'-thio-arabino-furanosyl Guanine and 2,6-Diaminopurine against Clinical Isolates of Human Cytomegarovirus**

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We presented at this meeting last year that 2'-fluoro-4'-thio-arabinofuranosyl derivatives of guanine (**1**) and 2,6-diaminopurine (**2**) showed potent and broad antiviral activity against herpesviruses including human cyto-megarovirus (CMV). In the present study, we tested antiviral effect of these compounds against seven recent clinical isolates of CMV *in vitro* by the plaque reduction method, comparing with the activity of ganciclovir (GCV). Both **1** and **2** were more active than GCV against all clinical isolates. In terms of ED_{50} values, the former were about five-times more active than GCV against six isolates tested as shown below. GCV-resistant 93-1 strain, another strain isolated from a patient who had received long-term GVC-treatment, was susceptible to **1** and **2**, (ED_{50} = 0.83 and 0.51 μM, respectively).

Compound	ED_{50} for 6 isolates (μM)		Max./Min.
	Mean	Rang	
1	0.38	0.31 - 0.49	1.60
2	0.33	0.27 - 0.34	1.24
GCV	2.21	1.18 - 4.78	4.39

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Identification of Novel Nucleotide Tetrahydrofuranyl Phosphonate Analogues With Potent Anti-HCMV Activity.

N. Nguyen-Ba, L. Chan, N. Turcotte, D. Barbeau , J. Bedard, L. Yuen, J.A.V. Coates, M. Ryan, and M. Sowa.
BioChem Therapeutic Inc., 275 Armand-Frappier Blvd., Laval, Quebec, Canada, H7V 4A7.
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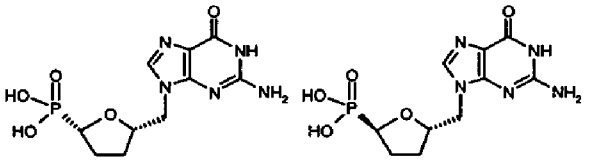
Further structure activity relationship studies of dioxolane phosphonate nucleotide analogue, from which the guanine derivative merged as a lead, allowed us to identify the guanine derivatives (cis and trans) of tetrahydrofuranyl analogues as potent anti-HCMV agents. Both compounds displayed *in vitro* and *in vivo* antiviral activity comparable to Ganciclovir and HPMPC. The compounds have been synthesized in enantiomerically pure manner from glutamic acid. Details of the synthesis and antiviral activity will be presented.



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DESIGN AND SYNTHESIS OF A NOVEL CLASS OF DIOXOLANE NUCLEOTIDE ANALOGS WITH ANTI-HCMV ACTIVITY.

N. Nguyen-Ba, M. Quimpere, W. Brown, N. Lee, H. Mitchell, S. Labine.
BioChem Therapeutic Inc., 275 Armand-Frappier Blvd., Laval, Quebec, Canada, H7V 4A7.

HCMV infections pose a serious problem for individuals whose immune system has been compromised by disease, such as AIDS or by medication as in organ transplant recipients. Although the current therapies for treatment of Human Cytomegalovirus (HCMV) are effective, they all suffer from serious toxic side effects such as nephrotoxicity (Foscarnet and HPMPC) and myelotoxicity (Ganciclovir); there is therefore a need for better HCMV drugs. We have designed a novel class of nucleotide analogs based on HPMPC by tethering the oxygen moiety of the terminal hydroxy group with the phosphonylmethoxy carbon. The outcome is a series of dioxolane nucleotides where the phosphonate group is directly attached to C-2 of the ring and the base is separated from the dioxolane ring by a methylene group. Preparation of purine and pyrimidine nucleotides was achieved by direct displayment of a leaving group or by a Mitsunobu coupling of the heterocylic base with a phosphonate sugar precursor. The guanine analogue showed marginal anti-HCMV activity in Flow 2002 cells. Details about synthesis and antiviral activity will be presented.

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Inhibition of Human Cytomegalovirus Proteinase by Salcomine Derivatives

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Salcomine derivatives are identified as potent, highly selective, HCMV proteinse inhibitors. The 50%inhibitory concentration(IC_{50}) of salcomine(RD3-0171) was 1.4 μM for HCMV proteinase whereas were >250 μM for trypsine, 206 μM for cymotypsine and > 250 μM for elastase. Other two derivatives(RD3-0174, RD3-0178) also inhibited HCMV proteinase under 2 μM and showed the same trend as salcomine against above three serine proteinases. The structure-activity relationship studies on these compounds showed that the pheny moiety and the spacer moiety (distance for two amines) play an important part on inhibitory activity for HCMV proteinase. Moreover salcomine inhibited viral growth of laboratory strain, AD169, and of three clinical isolate strains at the 50% effctive concentration (EC_{50}) within the range of 0.86 - 1.23 μg/ml. On the other side salcomine derivatives did not inhibite viral growth of HSV-1, HSV-2 and VZV even at the concentration of 10 μg/ml. Futhermore time-of-addition experiment showeded that salcomine derivatives act at late stage of HCMV replication, indicationg the mechanism of anti-HCMV action targeted proteinase inhibiton. These results suggested that Salcomine derivatives are pursuing as candidate drug for the chemotherapy of HCMV infections.

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Antiviral Activity of Lipid Prodrugs of Foscarnet and Ganciclovir Against the AD169 and Toledo Strains of Human Cytomegalovirus.

K. A. Aldern,[1] J.R. Beadle,[1] G.D. Kini,[1] C.A. Stoddart[2] and K.Y. Hostetler.[1]
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Human cytomegalovirus (HCMV) causes retinitis in AIDS patients and is the most common opportunistic infection after whole organ transplantation. We synthesized lipid prodrugs of foscarnet (PFA) and ganciclovir (GCV) and tested these prodrugs against the AD169 strain of HCMV. PFA and 1-O-octadecyl-2-O-methyl-*sn*-glycero-3-PFA (OMG-PFA) had EC_{50} values of 39.7 μM and 0.32 μM. Thus, OMG-PFA is 72 times more active than PFA. The EC_{50} of GCV and 3-hexadecyloxy-propane-1-phospho-GCV (HDP-P-GCV) were 1.6 μM and 0.45 μM (a 3.6-fold increase with HDP-P-GCV). We plan to use a SCID-hu Thy/Liv mouse model infected with a low-passage HCMV clinical isolate (Toledo) to evaluate these lipid prodrugs. In MRC-5 cells infected with HCMV Toledo, PFA inhibited viral replication with an EC_{50} of 50.3 μM versus 0.32 μM for OMG-PFA, while GCV and HDP-P-GCV were essentially equivalent with EC_{50} values of 0.6-0.7 μM. Preliminary data indicate that lipid prodrugs of PFA and GCV have increased oral absorption. Prodrugs of this type may be useful as oral antiviral agents.

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Novel Inhibitors of Human Cytomegalovirus (HCMV) Cascade Gene Expression

Jun Wu, Joe O'Neill and Miguel S. Barbosa, Virology Group, Signal Pharmaceuticals, Inc., San Diego, California 92121, U. S. A.

HCMV infection is a major cause of morbidity and mortality in immunocompromised patients. Problems with side effects and viral resistance of current therapy have made development of new and more effective anti-HCMV drugs a priority in antiviral drug research. HCMV infection of permissive cells in culture leads to an ordered sequential expression of viral genes which are divided into three kinetic classes: immediately-early (IE), early (E) and late (L). IE gene expression is essential for viral replication. These regulatory proteins present attractive targets for antiviral therapy since their inhibition should prevent viral replication without affecting the host-cell machinery. We established novel cell based assays to identify compounds that inhibit the HCMV cascade gene expression. Several compounds were identified and characterized. Compounds with antiviral activity were identified. Analysis of the inhibitor effect on the viral gene transcription characteristic of HCMV life cycle showed that the compounds acted by inhibiting the progression from IE to E phases of gene expression and viral production. The mechanism of action will be discussed.

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Inhibition of Proteinase Dependent Processing of the Scaffold Proteins of HCMV Capsid by Monocyclic β-Lactams

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Herpesvirus capsid assembly proceeds via a non DNA containing capsid with an internal scaffold core that is not present in mature virions. The scaffold is composed of the viral protease and assembly protein (AP) and proteolytic processing of these proteins is essential for capsid maturation in order to produce infectious virions. In human cytomegalovirus (HCMV), the protease is expressed as a precursor protein that undergoes autocatalytic cleavage to free the catalytic domain of the enzyme located in the N-terminal 256 amino acids. Our medicinal chemistry efforts towards the development of inhibitors of HCMV protease have generated a series of monocyclic β-lactam inhibitors of the catalytic domain. We have evaluated the activity of these inhibitors on the processing of the protease precursor as well as the processing of the assembly protein using cells transfected with the protease and AP, and in cells infected with HCMV. These protease inhibitors exhibited concentration dependant inhibition of the protease precursor processing as well as inhibition of the AP processing by the protease catalytic domain in transfected cells. When evaluated in infected cells, accumulation of the protease precursor and the assembly protein precursor could be observed in cells treated with these inhibitors. The results of this study suggest that these β-lactam inhibitors can inhibit both forms of HCMV protease in transfected and in infected cells. Experiments are in progress to correlate the inhibition of protease and assembly protein processing with effects on DNA encapsidation and maturation of capsids to form infectious virions following treatment with these inhibitors.

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Modulation of HCMV gene expression by isoquinolines.

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[3]Institut fur Klinische und Molekulare Virologie, Erlangen, Germany

The anti-HCMV potential of isoquinoline analogues was discovered using a plaque reduction assay with some of the more potent analogues in this series having IC_{50}s in the 0.005 µg/ml range with selective indices >100. These molecules were found to exhibit their highest antiviral activity when added to infected cells within 30 hours post-infection indicating a target acting at an immediate-early to early stage of virus infection. Utilizing virus specific MAbs and indirect immunofluorescence we determined that the isoquinolines were not interfering with virus infection but rather de novo synthesis of proteins expressed early in the infectious cycle was altered. We also determined, using a cell-based assay in which the HCMV MIEP promoter was linked to the luciferase gene, and expressed constitutively in HeLa cells, that the drug candidates were inhibiting transcription driven by the MIEP promoter. Plasmid transfection experiments using various subfragments of the MIEP linked to the CAT gene helped identify nuclear transcription factors affected by the presence of drug in the culture medium. In this regard we were able to decrease transcription enhanced by the 18 and 19 bp repeat elements found in the MIEP up to 80% at non-toxic drug concentrations. The correlation between reduced virus production in culture assays and the ability of the isoquinoline analogues to modulate important transcription factors necessary for the efficient replication of the virus, suggests that the targeting of such transcription factors may prove valuable in the design of novel antiviral agents.

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Pharmacokinetic Studies of Naphthyridine Analog HCMV Inhibitors and Discovery of Substituted Isoquinoline Analogs as Metabolically Stable Anti-HCMV Agents.

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Our recent effort in anti-HCMV program led to the identification of two novel classes of potent and selective anti-HCMV agents, the 1,6-naphtyridines and the isoquinolines. We were also encouraged by the fact that both lacked features that may be associated with poor oral bioavailability. However, during preliminary pharmacological evaluation, it was found that these compounds were not stable to the first pass metabolism. The metabolite was identified and found to be inactive in HCMV assay. Blocking the site of metabolism also abolished antiviral activity. However, based on SAR in naphthyridines and isoquinolines, we designed isoquinoline analogs and found that these compounds were much more metabolically stable both *in vitro* and *in vivo* and yet maintained anti-HCMV activity. The potential of these compounds for development will be evaluated.

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Antiviral agent glycyrrhizin stimulates human cytomegalovirus replication in retinal pigment epithelial cells

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[1]Institute of Med. Virology, [2]Center of Pediatrics, Department of Hematology and Oncology, J.W. Goethe-University, Frankfurt/M., Germany

Glycyrrhizin (GR) has been described as an antiviral agent against human cytomegalovirus (HCMV), herpes simplex virus type 2, hepatitis A and human immunodeficiency virus *in vitro*. GR has been also applied clinically to treat patients with chronic hepatitis or patients with AIDS. We used cultures of human eye glial (HEG) and retinal pigment epithelial (RPE) cells (established from postmortem eyes) as a model for study of GR effects on HCMV replication in cells types relevant for virus infection in patients with HCMV retinitis. In HEG cultures GR added after virus adsorption (90 min.) inhibited replication of several HCMV strains at concentrations ranging from 2 to 20 mM while in RPE cells only GR concentrations of 10 and 20 mM were inhibitory for HCMV. GR concentrations ranging from 0.2 to 2 mM stimulated HCMV replication in RPE cells. GR at a concentration of 1 mM showed maximum stimulatory effects increasing 20-fold numbers of RPE cells positive for HCMV immediate early (IE) and late (L) antigens and about 50-fold infectious virus titres. The increase of both IE and L phases of virus replicative cyclus was confirmed by RT-PCR measurements for HCMV IE and L mRNA. Ultrastructural investigations performed 3 days p.i. revealed in infected non-treated cultures only cells with viral nucleocapsids in nucleus while in infected cultures treated with 1 mM GR numerous cells with late phases of morphogenesis (i.e. enveloped virus particles and dense bodies in cytoplasma) were observed. Treatment of RPE cells with 1 mM GR resulted in dramatic increase of phagocytosis which could account for increased HCMV infectivity. The results showed that GR increases at therapeutic relevant concentrations HCMV replication in RPE cells. The data suggest that in addition to fibroblasts other cell types should be used to observe antiviral effects of novel drugs against HCMV.

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Phenotypic Assay for Drug Susceptibility of Clinical Isolates of Human Cytomegalovirus (HCMV). J. McSharry[1] and N. Lurain[2], [1]Albany Medical College, Albany, NY, and [2]Rush-Presbyterian Medical Center, Chicago, IL USA

The susceptibility of 25 HCMV clinical isolates to ganciclovir and foscarnet was determined by flow cytometry. MRC-5 cells monolayers were infected with HCMV-infected cells at an MOI of 0.01 to 0.1 infected cell per cell in the presence of various concentrations of ganciclovir (0 to 24 μM) or foscarnet (0 to 1600 μM). After 96 hr of incubation at 37°C, the cells were removed by trypsin, permeabilized with 90% methanol, treated with an FITC-labeled monoclonal antibody to an HCMV immediate early (IE) antigen, and the percent of antigen positive cells was determined by flow cytometry. The mean IC_{50} for ganciclovir susceptible clinical isolates was 4.31 μM (±1.92) and for foscarnet susceptible clinical isolates was 289.75 μM (±139.66). Drug resistant clinical isolates had IC_{50}s of >96 μM for ganciclovir and >800 μM for foscarnet. Similar IC_{50}s of the two drugs for these clinical isolates were obtained with the plaque reduction assay. The flow cytometry based assay is rapid, quantitative, and potentially automatable. These results show that this procedure can be used to determine the susceptibility of HCMV clinical isolates to foscarnet and ganciclovir and suggest that it may be useful for analysis of drug susceptibility of other viruses to other antiviral drugs. The flow cytometry assay should replace the plaque reduction assay for determining the drug susceptibility of clinical isolates of viruses that grow in tissue culture cells.

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A Point Mutation Within The CMV DNA Polymerase Confers Cross-Resistance to Foscarnet And Ganciclovir.

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2 Dept. of Medical Laboratory Technology, Stockholm University College of Health Sciences, Stockholm, Sweden.

Three antiviral drugs ganciclovir (GCV), foscarnet (PFA), and cidofovir (HPMPC), are available for clinical treatment. Therapy failure and resistance to these drugs have been reported. A CMV strain showing cross-resistance to PFA and GCV was isolated from a patient with AIDS who underwent sequential courses of therapy with HPMPC and PFA. By sequencing of the DNA polymerase (UL54), we found the amino acid alteration V-781-I. None of the mutations within the viral kinase (UL97), which confer resistance to GCV, was found. By marker transfer expriments with the Towne strain, the cross-resistance caused by the single (V-781-I) was confirmed. Comparative growth phenotype analysis between the patient's original isolate, recombinant virus, and Towne showed that this mutation was not responsible for the slow growth of the original strain. The recombinant strain showed the same kinetic protein expression and high infectious virus yields when tested in vitro as the laboratory strain Towne. We have performed molecular characterization of a mutation within the CMV DNA polymerase gene causing cross-resistance to PFA and GCV. This mutation was not associated with changed growth kinetics.

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Novel Benzimidazole-Resistant Human Cytomegalovirus Isolates Identify a Third Mutation in the UL89 Open Reading Frame. P.M. Krosky, S.M. Bigalke, R.G. Ptak, D.E. Murphy, L.B. Townsend and J.C. Drach, University of Michigan, Ann Arbor, MI 48109, U.S.A.

2,5,6-Trichloro-1-(ß-D-ribofuranosyl)benzimidazole (TCRB) and its 2-bromo analog (BDCRB) are potent and selective inhibitors of human cytomegalovirus (HCMV) replication. Incubation of strain AD169 in the presence of BDCRB selected virus with one or two mutations in UL89. A single mutation of Asp344Glu gave HCMV approximately 10-fold resistant to the benzimidazole nucleosides; a second mutation of Ala355Thr gave virus ~30-fold resistant (Underwood *et al.*, *J. Virol.*, 1998). Incubation of HCMV strain Towne with TCRB also selected virus with the Asp344Glu mutation; further incubation of this strain with 50 μM TCRB selected more highly resistant virus. One of these strains, designated C4, encoded the UL89 mutation plus a novel mutation in UL56 (Krosky *et al.*, Herpesvirus Workshop, 1997). We now report the characterization of other strains also selected by growth in 50 μM TCRB. Virus isolates designated SS and LA were purified by limiting dilution and were 20- to 30-fold resistant to TCRB and BDCRB in plaque and yield reduction assays. Isolate SS but not LA exhibited very small plaque morphology. Both isolates had the expected Asp344Glu mutation and both had an additional Asn353Ile mutation in UL89. They did not encode the Ala355Thr mutation in UL89 nor was there any mutation in UL56. These data confirm the importance of the UL89 gene product in the antiviral activity of the benzimidazole ribonucleosides and more firmly establish that TCRB and BDCRB interact with the portion of UL89 encoded by amino acids 344 - 355. Supported by grant U19-AI31718 from NIAID, training grant GM07767 from NIGMS, GCRC grant M01RR00042 for gene mapping computer facility, and by Glaxo Wellcome Co.

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The Frame-Shift Mutation in UL97 Gene of a Clinical Isolate of Cytomegalovirus from an AIDS Patient

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Division of Persistent & Oncogenic Viruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Kagoshima City, Japan

Cytomegalovirus (CMV) was isolated from an AIDS patient who developed progressive CMV disease in spite of treatment with ganciclovir (GCV). The isolate showed resistant to GCV with an IC50 of >6 uM when estimated by 50% plaque-reduction test. PCR-SSCP analysis revealed the difference of electrophoretic mobility in one fragment(Nucleotide No.1634 to 1990). When the fragment was sequenced, it was revealed that the first base at codon 594 (GCG) was deleted, resulting in frame-shift mutation of UL97 gene. Because of this frame-shift mutation, a new stop codon appeared at the 19th codon downstream of this point. Thus, the truncated UL97 gene product may not phosphorylate GCV so efficiently as the authentic one, resulting in conferring GCV-resistance on this isolate.

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Exploring the Mechanism of Action of 2-Hydroxymethyl-cyclopropylidenemethyl Purines by Selection of Drug-Resistant Human Cytomegalovirus. J.C. Drach, J.M. Breitenbach, K.W.-H. Feng and J.G. Jacobson, 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 the named compounds that have broad-spectrum antiviral activity (Qiu *et al., J. Med. Chem.*, 1998). The Z-isomers of the adenine (synadenol), guanine (synguanol), and 2-amino-4-cyclopropylamine purine analogs were particularly active against human cytomegalovirus (HCMV); IC_{50}'s ≅ 2 μM in plaque assays and IC_{90}'s ≅ 1-2 μM in yield assays. Both synadenol and synguanol were used to select drug-resistant HCMV by infecting human foreskin fibroblasts (HFF cells) with a low m.o.i. of Towne HCMV. Incubation in the presence of 4 μM compound was continued until cytopathology was apparent in most cells. Supernatants were used to infect new cultures of HFF cells in the presence of 8 μM concentrations of the compounds and the procedure repeated until virus would not grow or until drug cytotoxicity interfered (64 and 32 μM, respectively for synadenol and synguanol). 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 were less resistant to the other two compounds suggesting incomplete cross resistance among the three compounds. Additional phenotypic and genotypic characterizations of the drug-resistant isolates are in progress. This study was supported by grants U19-AI31718 and R01-CA32779 from N.I.H. and by funds from the University of Michigan.

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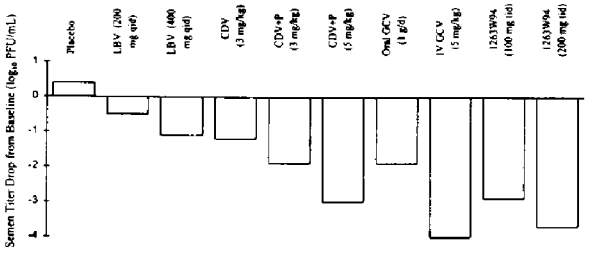
Utilization of Semen Titration to Assay In Vivo Antiviral Activity of Anti-CMV Drugs in Asymptomatic Subjects. DREW WL, MINER RC, MCMULLEN DJ, LALEZARI JP, GLUTZER E, WANG LH. UCSF Mount Zion Medical Center, San Francisco, CA, Quest Clinical Research, San Francisco, CA, Glaxo Wellcome Inc., Research Triangle Park, NC

Measurement of "body load" of virus has become important in antiviral reserach and therapy. Cultures or DNA assays of CMV in blood are rarely positive in patients with asymptomatic CMV disease and with CD4 count > 100 c/μL. We have used titration of CMV (plaque assay) in semen of HIV+ subjects to evaluate the dose-response of the *in vivo* antiviral activity of candidate anti-CMV compounds. In approximately 70% of HIV-infected gay men, their semen CMV cultures are positive and 20-30% of these have titer > 10^5 PFU/mL to allow evaluation of at least 4 log_{10}'s of reduction of CMV titer induced by potent anti-CMV compounds.



The relative potency of the *in vivo* anti-CMV activity as determined by log_{10} drop in semen CMV titer is consistent with the relative *in vitro* potency (e.g., IC_{50}). Interestingly, the extent of semen titer drop correlates with the clinical efficacy of two approved anti-CMV drugs, e.g., oral ganciclovir 1g tid vs. IV ganciclovir and cidofovir 3 mg/kg dose vs. 5 mg/kg dose for CMV retinitis. Whether this correlation can be applied to other anti-CMV agents will depend on pharmacokinetic factors such as ocular penetration. In conclusion, CMV titration in semen provides a model to evaluate the *in vivo* anti-CMV activity of new anti-CMV agents and can assist in the selection of appropriate dosages in subsequent clinical trials.

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Improved Method for the Detection of Ganciclovir in Plasma Using HPLC with Electrochemical Detection

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Ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)guanine) is used in the treatment of HCMV retinitis and for oral prophylaxis of HCMV infection in transplant patients. Several different HPLC methods have been published for its determination in plasma. Sample preparation typically consists of acid precipitation or ultrafiltration for removal of serum proteins prior to analysis. Most HPLC separations are performed on reverse phase C18 columns in aqueous buffers while some inlcude ion pair reagents or a small percent of organic modifier. Limits of detection as low as 0.025 mg/L have been reported for UV and 0.010 mg/L for fluorescence methods. Similar results have been reported for acyclovir, valacyclovir and penciclovir. We present here a sensitive HPLC method using a 4.6 x 150 mm Beckman Ultrasphere ODS column, 60 mM sodium phosphate buffer, pH 3.05 isocratic at 1 ml/min with coulometric detection at 700 mV and detection limits of 0.001 mg/L for ganciclovir, representing a 10-fold increase in sensitivity. Data demonstrating the utility of this detection method for use with other guanosine-based antivirals will also be presented.

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The Effect of Intraperitoneal Injection of 1-O-octadecyl-*sn*-Glycero-3-Foscarnet and its Carboxymethyl Ester on Mortality and Serum Calcium Levels in Mice.

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PFA is approved for the treatment of AIDS-associated cytomegalovirus infection. Because PFA is poorly absorbed orally, we synthesized 1-O-octadecyl-*sn*-glycero-3-foscarnet (ODG-PFA) and its carboxymethyl ester (ODG-PFA-OMe). These prodrugs were 57- and 28-fold more active than PFA in HCMV-infected cells. To evaluate acute PFA toxicity, we gave 200 mg/kg of each of the prodrugs intraperitoneally to mice. Serum calcium levels in control mice injected with saline were 8.4 ± 0.5 mg/dl throughout while animals treated with ODG-PFA were noted to have serum calcium levels of 5.6 to 6.4 mg/dl two hours following injection. Three to six hours after injection of ODG-PFA a 70% to 88% mortality was noted. In contrast, mice receiving 200 mg/kg of ODG-PFA-OMe had only a transient drop in serum calcium. No deaths or acute toxicity was seen in mice treated with ODG-PFA-OMe. We conclude that the acute toxicity of ODG-PFA is due to hypocalcemia which can be abolished by attaching a methyl ester group to the carboxyl moiety. ODG-PFA-OMe is well absorbed orally and is well-tolerated acutely in mice.

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The Use of Electroretinography to Study MCMV Induced Retinitis in the SCID Mouse. M. Garneau*, G.T. Bolger, C. Bousquet, P. Kibler and M.G. Cordingley, Bio-Méga Research Division, Boehringer Ingelheim (Canada) Ltd. 2100 rue Cunard, Laval, Québec, Canada H7S 2G5

Cytomegalovirus associated retinitis is a major cause of vision loss in the AIDS patient. A safer and more effective antiviral treatment that would prevent development of retinitis represents an ongoing effort in research. While histopathology or viral titration are the preferred methods to evaluate retinitis in murine cytomegalovirus (MCMV) models, we investigated the use of electroretinography (ERG) as a noninvasive technique to measure the functional activity of the retina following MCMV anterior chamber inoculation. While under anesthesia, a volume of 1.9 µl containing either control salivary gland homogenate or 400 pfu of salivary gland passed, Smith Strain MCMV was injected in the anterior chamber of the right eye of 6 to 8 week old SCID mice. At various times post inoculation, scotopic ERG recordings were obtained from the injected eyes. The wave amplitudes were compared. In mice inoculated with control salivary gland homogenate, no change in A or B wave amplitude was noted, up to day 21 post inoculation. Both the A-wave and the B wave amplitude recorded from infected eyes were significantly reduced ($p < 0.05$; ANOVA SNK multiple comparison) as of day 10 post inoculation. When antiviral therapy using HPMPC (5 mg/kg/day s.c. once daily for 5 consecutive days) was started 3 hr post inoculation, no significant changes were observed in the different ERG parameters up to day 21 post inoculation. Viral titration in these eyes revealed higher viral titer in the injected eyes of non treated animals compared to HPMPC treated animals. Histopathological examination of the eyes revealed a good correlation between the ERG B wave amplitude and the extent of retinal desease. These results suggest that ERG recording represent a valuable noninvasive technique to measure retinal desease following MCMV anterior chamber inoculation in SCID mouse.

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Kinetic Studies of the Inhibition of Influenza Neuraminidases by GS 4071. D.B. Mendel[1], P.A. Escarpe[1], C.Y. Tai[1], M.A. Williams[1], W. Lew[1], H. Wu[1], C.U. Kim[1], G. Laver[2], and B.J. Graves[3]. [1]Gilead Sciences, Inc., Foster City, CA, USA, [2]Australian National University, Canberra, Australia, and [3]Roche Discovery Welwyn, Welwyn Garden City, Hertfordshire, U.K.

GS 4071 (Ro 64-0802) is a novel, potent, and selective inhibitor of the influenza neuraminidases. GS 4071 derives much of its binding energy through hydrophobic interactions with the region of the enzyme active site normally occupied by the glycerol side chain of the natural substrate, sialic acid. In this study we have investigated the kinetics of inhibition of the influenza neuraminidases by GS 4071 in order tc more fully understand the inhibitor-enzyme interaction. Our results demonstrate that the kinetics of neuraminidase inhibition by GS 4071 are biphasic, indicating that GS 4071 is a slow-binding inhibitor of the influenza neuraminidases. Structural studies suggest that the slow-binding property of GS 4071 is likely due to a re-orientation of the Glu276 side chain in the enzyme active site. These results are consistent with the earlier demonstration that the re-orientatior of the Glu276 side chain is necessary to optimize hydrophobic interactions between GS 4071 and the enzyme.

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Synergistic In Vitro Anti-Influenza Virus Effects of the Neuraminidase Inhibitor GS4071 and Ribavirin. J. H. Huffman, A. Morrison, T. Syndergaard, and R. W. Sidwell. Institute for Antiviral Research, Utah State Univ., Logan, UT, U.S.A.

The carbocyclic transition state sialic acid analog, GS4071, a potent neuraminidase inhibitor, has previously been shown to have striking in vitro inhibitory effects on a spectrum of influenza A (H1N1, H3N2) and B viruses. An experiment was run to determine if this compound and ribavirin, which inhibits influenza virus replication by other mechanisms, will act in a synergistic manner against the virus when used in combination. Seven doses of GS4071, ranging from 0.031 to 2 µM were run with seven doses of ribavirin ranging from 0.39 to 100 µM in a checkerboard-type experiment using influenza A/Washington/05/96 (H3N2) virus in MDCK cells. Inhibition of viral cytopathic effects were determined by microscopic examination and neutral red dye uptake, and, in a second experiment, AlamarBlue™ uptake using a computer-driven automated fluorimeter. Depending upon the assay method used, the EC50 values for GS4071 used alone ranged from 0.1 to 3 µM, and for ribavirin used alone ranged from 19 to 28 µM. When used at every dosage of ribavirin, the GS4071 EC50 dropped from 2- to >3-fold; similarly, at every dosage of GS4071, the ribavirin EC50 declined from 8- to >49-fold. Analysis of the data using either the Berenbaum Fractional Inhibitory Concentration method or the Prichard and Shipman computerized 3-D model, each indicated a strong synergistic effect with this drug combination. (Supported by Contract NO1 AI-35178 from the Virology Branch, NIAID, NIH).

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Activities of zanamivir (GG167) and GS4104 in a series of influenza A virus animal models.

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Influenza neuraminidase inhibitors, typified by zanamivir, have been shown to be safe and effective antivirals in both animal models and experimental influenza virus infections in man. Primary testing has relied on the predictability of *in vitro* enzyme assays, *in vitro* live virus assays and ultimately *in vivo* mouse models. We report here the efficacy of zanamivir and GS4104 given by various routes, in both lethal and non-lethal mouse models of influenza A infection. We have attempted to correlate the efficacy in these murine models with the efficacy demonstrated in the ferret model. These data indicate discrepancies between efficacy profiles of orally administered neuraminidase inhibitors in non-lethal and lethal mouse models of influenza A infection. We propose from these studies that the non-lethal lung virus titre model in the mouse is more predictive for activity in the ferret, than the mouse survival model.

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***In vitro* and *In vivo* Inhibitary Activity of Influenza A virus by Phosphorothioate Oligodeoxynucleotides,**

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In order to develope antisense oligonucleotides as potential antiviral therapeutics against influenza A virus, phosphorothioate oligodeoxynucleotides (S-ODN) targeted to the 3' and 5' end sequences of influenza A virus RNAs were synthesized. Antiviral activity of these S-ODNs was evaluated by hemagglutination titer assay using cultured MDCK infected by A/JingFang/86-1(H1N1). Oligonucleotides complementary to 5' terminal conserved sequence, IV4#, exhibited the most potent antiviral activity, with reduced hemagglutination titer about 50% at concentration of 1μmol/L, and over 70% at 10μmol/L. The antiviral activity of IV4# was in a dose- and sequence-dependent manner. Incorporation of hydrocarbon chains onto IV4# showed no increased inhibition activity. In the presence of lipofectin IV4# and its hydrocarbon chain linked analog exhibited markedly increased antiviral activity, with inhibition activity from 56.2% to 75% and from 30% to 57.5% respectively in concentration of 1μmol/L. The effect of IV4#ODN on replication of Influenza A virus in embryonated eggs and BALB/c mice was also studied. At dose of 50μg/egg, IV4# prevented proliferation of A1/JingFang/86-1(H1N1)(10^5PFU/egg) completely in embryonated SPF eggs. Intranasal administration once daily for 5 days of IV4# (100μg/mice) significantly decreased HA titer(expressed as Log_2)of mice lung homogenate from 2.3 to 1.9.

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Anti-influenza A virus activities of antisense phosphorothioate oligonucleotides in mice depend on presence of cationic liposome and route of administration.

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[1]Rational Drug Design Laboratories, [2]Department of Microbiology, Fukushima Medical College, [3]Department of Industrial Chemistry, Chiba Institute of Technology, Japan

Here, we demonstrate the first successful *in vivo* antiviral activity of antisense administered by intravenously in experimental respiratory tract infections induced with influenza A virus. PB2-as and PA-as are novel antisense phosphorothioate oligonucleotides with PB2 and PA target sites of influenza A virus RNA polymerase and are potent inhibitors of influenza A virus replication *in vitro*. In this *in vivo* study we have examined the antiviral effects of PB2-as and PA-as in a mouse model o f influenza A virus infection. Balb/c female mice were infected with treatments given intranasally at 100 50% lethal doses (LD50s) of influenza A virus (A/PR/8/35). PB2-as and PA-as encapsulated by several liposomes (DOTAP, Tfx-50, Tfx-10, Coatsome) were administrated by intranasal, intraperitoneal and intravenous routes with various doses of 5 to 100 mg/kg for 4 days postinfection. In comparison with liposome-free PB2-as, liposomal PB2-as treated by an intravenous route significantly prolonged the mean survival time in days (MSD) and increased the survival rates with dose dependent manner. In addition, the liposomal PB2-as significantly inhibited the virus growth in lung tissues and reduced the pulmonary consolidations. These results indicate that PB2-as targeted to influenza A virus PB2 gene can effectively inhibit influenza A virus.

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Anti-influenza viral glycoprotein from the roots of *Isatis tinctoria* L.

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The roots of *Isatis tinctoria* is a well known Chinese herb, which was found to be effective for treatment of "cold" syndromes hundreds years ago. Now, this herb has been used clinically to prevent the influenza virus and hepatitis virus infections in China. However, the active substances have not been identified. Therefore we purified and characterized major anti-influenza virus active substance from the roots of *I. tinctoria*. High molecular fraction (IW-1) from the roots of *I. tinctoria* was shown to have anti-influenza viral activity by the virtue of cell culture method and by determination of survival virus in nasal cavity and BAL of mice infected with mouse-adapted A/PR/8/34 virus. When IW-1 was purified by gel-filtration on Sephacryl S-200 column, anti-influenza viral activity was observed in the subfraction of MW 7000 (IW-1-c). IW-1-c contained 17.5% of carbohydrate and 21.5% of protein. By elementary analysis, 28.6% of chloride ion was also found in IW-1-c. Eighteen kinds of amino acid containing arginine as major component were detected in the protein moiety of IW-1-c. Methylation analysis of IW-1-c showed that carbohydrate portion of IW-1-c was mainly composed of →4Glc1→ and →6Glc1→. Pullulanase digestion disappeared anti-influenza viral activity of IW-1-c. This result indicates that α-(1→6)-Glc chain of IW-1-c some involves in its anti-influenza viral activity. IW-1-c gave single protein and carbohydrate band by SDS-PAGE. These results indicate that the roots of *I. tinctoria* contains anti-influenza viral glycoprotein which is effective *in vitro* and *in vivo*.

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The Mechanism of Action of Arbidol Against Influenza Virus. Selection and Characterization of Arbidol-Resistant Mutants.
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A new anti-influenza drug arbidol (1 methyl-2-phenyl-thiomethy-3-carbotoxy-4-dimetylaminomethyl-5-hydroxy-6-bromo-indolhydrochloride monohydrate) was developed in the Russian Research Chemical –Pharmaceutical Institute. Arbidol inhibits early stages of influenza A and B reproduction. Using fluorescence dequenching assay and haemolysis , arbidol was shown to inhibit membrane fusion in vitro both between virus and the plasma membrane at pH=5,0 and between virus and the membrane of endocytic vesicles. The arbidol effect upon replication reassortants between influenza virus A/Singapore /1/57 and A/Weybridge/27 was studied. One group of viruses that have HA of Weybridge was very sensitive to arbidol, the second group of reassortant viruses which were less sensitive to inhibition by arbidol, contained HA of A/Singapore/1/57 . There was no correlation between sensitivity to arbidol and any other gene. The most sensitive to arbidol reassortant was chosen for study of arbidol-resistance and arbidol-resistant mutants were obtained by passing viruses in MDCK cells in the presence of increasing drug concentrations. Resistance of mutants obtained were confirmed in cell-ELISA and in plaque assay . Mutants selected for resistance to arbidol promoted membrane fusion at higher pH (0,2-0,4) than wild type virus. Arbidol inhibited haemolysis induced by wild type virus, but did not inhibit the haemolysis induced by arbidol-resistant mutants. To determine mutations responsible for resistance the HA genes of wild-type and arbidol-resistant mutants were sequenced. All mutants had mutations in HA2, but in different positions. The data obtained indicate that target of arbidol is the HA and the mechanism of arbidol action is connected to inhibition of a process of membrane fusion.

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IN VITRO COMBINED APPLY OF PROTEOLYTIC INHIBITORS WITH UNITHIOLUM.

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Proteolytic systems as well as disulphide bonds have an extreme significance in the virus-cell interactions. -S-S- bonds are stabilising element for proteins incorporates in the viral surface as well as for receptor proteins of surface membrane of sensitive cells. In our previous research there were shown antiviral activity of proteolytic inhibitors and Unithiolum (UT). The combination of these preparations were investigated in present research. Unithiolum, concentration 5,0 mg/ml, Ambenum (Am) and E-aminocaproic acid (E-ACA), concentration 2,5 mg/ml inhibited the viral reproduction of influenza strain A/PR/8/34 (H1N1) in tissue culture of chorioallantoic membranes of chick embryos. Combined apply of Am or E-ACA with UT was more effective than application any of these preparations lonely. Influenza virus B/Leningrad/17/86 was more sensitive to UT (2,5 mg/ml) than to Am (3,0 mg/ml) or to E-ACA (5,0 mg/ml). Combined usage of UT with Am or E-ACA on influenza virus B/Leningrad/17/86 was less effective than application any of these preparations lonely.

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Mechanism of specific inhibition on respiratory syncytial virus replication by benzoditiin compound, RD3-0028

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A benzoditiin structure compound, RD3-0028, was found to be a potent inhibitor on respiratory syncytial virus (RSV) replication. Its action is specific, in that no activity is seen against other viruses. RD3-0028 was capable of inhibiting growth of RSV and improving pathologic change for interstitial pneumonia in immunosuppressed mice when delivered by small particle aerosol. RD3-0028 had no direct virucidal effect on RSV. Time of drug addition studies indicated that the mechanism of antiviral activity is mediated late in the RSV replication cycle since this compound retained full antiviral activity when added up to 10 hours after infection of cell monolayers at an MOI of 3. This compound also inhibited syncytium formation even when added at the late stage. Moreover, five independent isolates of RSV long strain were selected for growth in RD3-0028 (5 - 20 μg/ml). These resistant viruses were > 160-fold less sensitive to RD3-0028 than long strain. The F gene segment of each of these viruses was sequenced and in each case the mutant RNA segment contained at least one sequence alteration, converting asparagine 276 to tyrosine (F protein numbering). These results suggest RD3-0028 inhibits RSV replication by interfering with viral protein synthesis, intracellular processing of the RSV fusion protein or a step immediately thereafter .

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Potential of the IMP Dehydrogenase Inhibitors for Antiviral Therapies of Poxvirus Infections D.F. Smee* and J.W. Huggins. U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD 21702-5011

Although smallpox virus has been eradicated from the earth, concerns about the spread of other poxviruses is increasing. Monkeypox virus has recently reemerged in the Democratic Republic of the Congo, transmitted person to person Molluscum contagiosum is a problematic skin infection in AIDS patients which is caused by another pox virus. We evaluated a number of antiviral agents for activity in cell culture against camelpox, cowpox, monkeypox and vaccinia viruses. The IMP dehydrogenase inhibitors ribavirin and mycophenolic acid (MPA) were inhibitory, with MPA being 10-fold more potent than ribavirin. A 50% inhibition of plaque formation in both Vero and mouse 3T3 cells was achieved with MPA at 1-2 μM, whereas ribavirin inhibited at 200 μM in Vero cells and at 10 μM in 3T3 cells. The inhibition of cowpox virus by MPA and ribavirin in the two cell lines was correlated to a reduction in cellular guanosine triphosphate levels (a result of inhibition of cellular IMP dehydrogenase). Adding guanosine to drug-treated cultures reversed the anti-poxvirus activity in a dose-dependent manner. In a lethal cowpox intranasal infection in mice, ribavirin and MPA at doses of 25, 50, 100 and 200 mg/kg/day (given in divided daily doses) did not prevent mortality, although ribavirin delayed death by 1-2 days.

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A CYCLIC UREA DERIVATIVE (CU), AN IN VIVO EFFICIENT INHIBITOR OF FLAVIVIRUS REPLICATION

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Compound CU, synthesized by D. Sidzhakova et al. showed a pronounced inhibitory effect on the replication of bovine viral diarrhoea virus (BVDV) in cell cultures. A selectivity ratio ($CGIC_{50}/MIC_{50}$) exceeding 1560 was recorded in calf trachea (TC) cell line. The compound manifested an activity against a broad spectrum of BVDV strains tested, including wild and vaccinal atenuated strains. The sensitive to CU period in BVDV replicative cycle embraced both the latent period and the exponential phase. A 90-min delay in viral RNA synthesis was revealed in the one-step virus growth cycle setup. This effect was not confirmed in the case of the 17D strain of yellow fever virus replication in human adenocarcinoma cells. Only a 30 per cent inhibition of infectious YFV yield was found (on the 48th h) when CU was added similtaneously with virus inoculation. In vivo testing of CU in mice experimentally infected with 10 LD_{50} if the Sophin strain of tick-born encephalitis virus (TBEV) demonstrated a marked protective effect. The optimal treatment course, a daily dose of 150 mg/kg (devided in two intakes) administered subcutaneously during 5 days post infection, resulted in 40 per sent survivors. The compound was effective also in calves experimentally infected (intravenous inoculation of $5x10^7$ $CCID_{50}$) by the Kableshkovo strain of BVDV. CU was administered orally (two intakes) in daily doses 1, 3 and 10 mg/kg. The treatment with CU did not influence the seroconversion checked by the virus-neutralizing antibodies titer. Moreover, CU showed a marked effectivity administered in calves with natural mucosal disease. The field trial was realized on test-groups of 20 healthy calves each in separate boxes, three sick animals with well expressed clinical picture and serologically confirmed etiology being introduced in each box. CU was administered orally (by the milk), 10 mg/kg daily (a 12 h dose interval) during 9 days, prevented the appearance of the characteristic symptomatology (a two-peaks curve fever, anorexy, bronchitis, etc.). All animals in the placebo (control) group developed a well manifested clinical picture of mucosal disease. These data supplemented by the very favourable results of the toxicolology (one-month) study characterize CU as a very prospective anti-flavivirus chemotherapeutic agent.

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Glycyrrhizic acid as inhibitor of Marburg virus reproduction.

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Marburg virus (MBV) is etiologic agents of haemorrhagic disease in humans, with an extremely high pathogenicity. This viruses fit well into the modern concept of emerging and re-emerging viral diseases. Well known antiviral substances, such as interferon and ribavirin, have exhibited only low inhibitory activity on experimental MBV infections. We found that Glycyrrhizic acid (GA) and its derivatives Glyceram (monoammonium salt of GA) and Niglizin (penta-o-nikotinate of GA) provide clear inhibitory effect on MBV multiplication in virus-infected Vero cell culture. The plaque count is reduced to 10% -1% of the control when 1000 μg/ml GA or its derivatives is added to the overlay.

Compound	CD_{50} (mM)	ID_{50} (mM)	IS
GA	3.9	0.47	8.3
Glyceram	8.47	0.28	30.25
Niglizin	1.93	0.042	45.96

We define also the index selectivity (IS) as the ratio of the concentration of a test compound producing a 50% decrease in noninfected cell viability (CD_{50}) to the drug concentration causing a 50% plaque reduction (ID_{50}). Inhibitory effect of GA derivatives is not connected with induction of cell resistance to virus, since preliminary glyceram addition to cells during of 24 hours (with the subsequent removal of a preparation) does not effect on cell sensitivity to virus. Experiments with delaying the addition of compound at various time after the exposure of Vero cells to MBV shown that antiviral activity of GA related with inhibition of postbinding early stages of MBV reproduction.

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Fatty acid analogs as inhibitors of arenavirus replication.

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Two major types of fatty acylation of viral proteins, the incorporation of palmitic acid (C16:0) or myristic acid (C14:0), has been shown to be essential for diverse processes during infection with retroviruses and other clinically important viruses. Consequently, protein palmitoylation and myristoylation became a novel and promising target for antiviral agents. In the present study, fatty acid analogs have been evaluated in vitro as potential inhibitors of Junin virus (JV), agent of Argentine hemorrhagic fever, and the antigenically related arenavirus Tacaribe (TV). The tested compounds were 2-hydroxytetradecanoic acid (2-hydroxymyristic acid 2HM), 12-methoxydodecanoic acid (13-oxamyristic acid 13OM) and 2-hydroxyhexadecanoic acid (2-hydroxypalmitic acid 2HP). The three analogs inhibited the multiplication of JV, strains IV4454 and XJ, and TV in a dose dependent manner, as determined by a virus yield inhibition assay in Vero cells. The IC_{50} values ranged from 8 to 40 μM, with 2HM and 13OM as the most active inhibitors. Both extracellular and intracellular virus yields were similarly reduced. Neither compound was toxic to Vero cells in an MTT-based assay at concentrations up to 100 μM. A limited pulse of only 4h with 2HM or 2HP after several cycles of infection was enough to inhibit virus production, probably due to their effect on a late event in JV infective cycle. By immunofluorescence staining and by rediolabeling with [^{35}S]-methionine followed of immunoprecipitation it was shown that the fatty acid analogs did not inhibit viral protein synthesis but markedly blocked the presence of the envelope glycoprotein GP38 at the cell surface, preventing the production of infectious virions.

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Cidofovir is a potent inhibitor of murine polyoma virus-induced hemangioma formation in rats

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We evaluated, in newborn rats, the effect of the acyclic nucleoside phosphonate analogue cidofovir against the formation of hemangiomas induced by an abortive infection with murine polyomavirus (mPyV). Untreated, infected rats developed cutaneous, peritoneal and cerebral hemangiomas, associated with severe hemorrhage and anemia leading to death within 3 weeks post infection (p.i.). Subcutaneous treatment with cidofovir at 25 mg/kg, once a week, resulted in a complete suppression of hemangioma development and associated mortality when treatment was initiated at 3 days p.i. (100% survival as compared to 0% for the untreated animals). Cidofovir still afforded 40% survival and a significant delay in tumor-associated mortality when treatment was started at a time at which cerebral hemangiomas were already macroscopically visible (i.e., 9 days p.i.). Furthermore, in those animals, almost no hemorrhages were noted, and remarkably fewer and smaller hemangiomas developed as compared to the untreated animals. A semi-quantitative PCR for murine polyoma virus VP1 DNA revealed no viral replication except for some limited replication in the brain. An antitumor effect, rather than inhibition of viral replication, may be the reason for the inhibitory activity of cidofovir in this model. Indeed, (i) cidofovir was found to inhibit the development of established cerebral hemangiomas and (ii) the compound resulted in complete protection, even when treatment was initiated at 3 days p.i., a time at which transformation of the endothelial cells by the virus must have been initiated. The potent activity of cidofovir on vascular tumor growth in this model was accompanied by no marked toxicity for the host.

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IN VITRO INFECTION WITH PARVOVIRUS B19

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Parvovirus B19 is the causative agent of a variety of different manifestations, ranging from asymptomatic infection to severe clinical symptoms. The most prominent disease is the harmless childhood illness *erythema infestiosum* ("fifth disease"), but B19 can also cause persistent or chronic arthritis, *hydrops fetalis*, aplastic crisis and other symptoms. Until now there is no antiviral therapy for B19-infection, in some cases pooled immunoglobulins leads to improvement or resolving the symptoms. The basis for the great variety of B19-correlated illnesses is yet poorly understood. Most symptoms correlate to the high cell tropism of the virus. Permissive cells for viral replication are proliferating and differentiating, erythroid precursors in human bone marrow. Most certainly the target cells are in the erythroid lineage from BFU-E to erythroblasts.Viral entry is mediated through the Parvovirus B19 receptor, the P-blood group antigen (globoside), which is present on a variety of cells. So the high cell tropism can not exclusively be regulated by the receptor. To further examine the cell tropism we tried to produce recombinant viruses, consisting of a reporter DNA enclosed by the B19 capsid. The reporter DNA, functioning as the genome of the recombinant B19 virus, is derived from the autonomous, apathogenic parvovirus LuIII, with the coding region of the LuIII capsid proteins exchanged for the coding region of the green fluorescent protein (GFP). By encapsidating this LuIII-GFP-construct (pGLuP38GFP) in the B19 capsid it is possible to infect different cell types, that carry the B19-receptor. Thus new data about the cell tropism of parvovirus B19 and therefore also of the course of infection and the arising symptoms can be obtained. Better understanding of the cell tropism will consequently lead to progress in antiviral attack against parvovirus B19. Furthermore this system could be useful in the development of gentherapeutic targeting of primitive hematopoetic precursors.

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Generation of Human Monoclonal Antibodies against Parvovirus B19

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Antiviral antibodies appear to represent the principal means of defence against B19 Parvovirus infection. Therefore persons suffering from acute or persistent B19 Parvovirus infection, e.g. immunosuppressed persons, may be successfully treated with immunoglobulin-preparations containing anti-B19 antibodies. However, the use of human monoclonal antibodies (mAbs) to B19 virus should be more effective as a therapeutic agent. Furthermore, human mAbs against B19 can provide information for the field of vaccine development and virion organisation. To generate human mAbs, sera from 55 donors were tested for the presence of IgG antibodies (Abs) against B19-proteins. Sera were screened by ELISA with two recombinant proteins, NS-1 and the unique part of the minor capsid-protein VP-1. Blood samples were obtained from individuals with high titres of Abs. mAbs were produced via fusion of Epstein-Barr virus-transformed lymphocytes with human-mouse heteromyeloma cells SHM-D33. 35 % (17 of 55) of the individuals tested showed VP-1 specific antibodies in the serum and 5 % (3 of 55) had antibodies against NS-1. From two healthy donors with the highest serum titres, three mAbs were generated: one mAb (IgG3, kappa) against NS-1 and two mAbs (both IgG1, lambda) against the unique region of VP-1. Our experiments demonstrate the feasibility of generating human mAbs against B19 proteins from healthy donors who have no evidence of acute or chronic parvovirus infection. In addition, it is discussed that circulating Ab-producing cells in these individuals, which usually disappear after a convalescent period, may result from permanent stimulation by latent infection or by repeated inapparent B19-reinfection.

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Mutational Analysis of The RNA Involved In Ribosomal Frameshifting In Barley Yellow Dwarf Virus. D. Sung & H. Kang, Kumho Life & Environmental Sciences Laboratory, 572, Ssamgam-dong, Kwangsan-ku, Kwangju, Korea

Plant viruses use non-standard decoding events, ribosomal frameshifting and stop-codon readthrough, to synthesize essential proteins from the overlapping genes. In order to understand the mechanism of frameshifting and plant virus replication, site-directed mutagenesis is used to investigate the functional aspect of the RNA that plays an important role in ribosomal frameshifting in plant viruses. A simple and efficient *in vitro* frameshifting assay using luciferase reporter gene was developed. Various DNA sequences comprising the shift site and the downstream enhancer were cloned into the pGEM-LUC vector to test the effect of downstream RNA sequence and/or the structure on ribosomal frameshifting. *In vitro* transcription using SP6 RNA polymerase, and translation using either rabbit reticulocyte lysate or wheat germ extract yielded different level of active luciferase dependent on the downstream RNA sequences. The frameshifting efficiencies were varied in between 20 to 40 % depending on the RNA sequence. To test the RNA sequence/structure dependent frameshifting *in vivo*, a fragment of the pGEM-LUC carrying the BYDV frameshifting signals were cloned into the yeast expression vector, pAS2-1, and the *in vivo* effect of the RNA sequence/structure on the frameshifting was investigated. Our analyses reveal that frameshifting occurs in response to the RNA sequence, and that the downstream RNA structure is required for efficient frameshifting in plant virus.

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Application of Antisense and Ribozyme In Controlling The Viral Replication. H. Kang & D. Sung, Kumho Life & Environmental Science Laboratory, 572, Ssangam-dong, Kwangsan-ku, Kwangju, Korea

In an attempt to develop efficient way to inhibit the propagation of plant viruses, antisense RNA and ribozyme targeted to the frameshifting site of the plant viruses were designed and their activities were tested using the luciferase reporter system. A 17mer RNA complementary to the RNA sequence including the frameshifting site of the Barley Yellow Dwarf Virus (BYDV) completely arrests the translation of luciferase gene. This inhibitory effect is dependent on the concentration of the antisense RNA. A 36mer hammerhead ribozyme was designed to cleave the RNA at the frameshifting site. Translation of the luciferase gene is arrested by the ribozyme. In order to test the efficacy of the antisense and ribozyme *in vivo*, a yeast expression system using pYES2 vector was designed. The genes coding the antisense and/or ribozyme were cloned into the Pvu II / Sph I site of the pYES2, which is expressed under the control of the GAL1 promoter. The pYES2 vector was co-transfected into the yeast with the pAS2-1 vector carrying the frameshifting assay cassette of the BYDV. Analysis of the protein products by Western Blotting reveals that antisense and ribozyme completely inhibit the translation of the messenger RNA beyond the target site. Our results indicate that frameshifting site in viruses is a good target site for antisense and ribozyme, which is a potential way to inhibit the viral propagation.

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Capsid and RNA stabilisation of the Oral Polio Vaccine

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The oral polio vaccine is thermolabile. Two inactivation mechanisms are involved in this process : a degradation of the viral capsid and a denaturation of the viral RNA. After incubation at elevated temperatures (42-45°C range), pirodavir, a capsid-binding compound produced by the Janssen Research Foundation, inhibits the antigenic N (native) to H (heated) conversion of the viral capsid. However, the infectivity of the virus was still significantly reduced. Since the viral capsid is stabilized by pirodavir, we examined separately the infectivity of the viral RNA in transfection experiments. Pirodavir has little effect on viral RNA thermodenaturation. Crainic et al., (1996) showed that heavy water (D_2O) enhances significantly the stability of poliovirus, probably by a mechanism which strengthen hydrogen bounds in the viral structure. We confirmed these results. Moreover, we were able to show that D_2O mainly prevented viral RNA thermodenaturation. When a combination of pirodavir and D_2O was used, the thermostability of the vaccine was further increased.

Crainic R., Wu R., Otelea D., Georgescu M.M., Delpeyroux F., Guillot S., Balanant M. & Tardy-Paint M. (1996). Dev. Biol. Stand. Basel, Karger. 87, 161-166.

Oral Session VIII: Mini-Symposium – The Use of Viral Vectors in Gene Therapy

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Drug Discovery Assays for Hepatitis C Virus: Development of a High Throughput Assay for the NS3 RNA Helicase.

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The coupled nucleoside triphosphatase/RNA helicase activity of the Hepatitis C virus NS3 protein is essential for viral replication and thus may represent a target for development of antiviral agents. Previously, we described a high-throughput screening assay for the ATPase activity (Antiviral Research, 30, 25, 1997) using a soluble truncated form of this protein. We have investigated 2 formats for high-throughput assay of the RNA helicase utilizing an RNA-DNA hybrid substrate (32 and 25 nucleotides, respectively, with a [^{32}P]-label on the DNA). In one format the substrate was immobilized in a microtiter well and unwinding activity yielded a decreasing signal. We observed low activity of the enzyme on this fixed amount of substrate, which may be due in part to slow diffusion of the enzyme to the plate surface as well as steric hindrance limiting access of the enzyme to the short substrate molecules. An increasing signal was obtained by allowing the enzyme to unwind the hybrid substrate in solution and capturing the released [^{32}P]-DNA oligomer by hybridization to a complimentary 25 nucleotide DNA immobilized in the well. We previously observed in a gel-shift assay that the helicase is incapable of unwinding a blunt DNA-DNA substrate, and thus the captured product represents a kinetic dead-end. The capture-format assay was linear up to at least 450nM enzyme and 10μM hybrid substrate, for at least 30 minutes at 37° C. The unwinding was completely dependent on ATP, with increasing activity up to 1mM. The reaction was inhibited by poly-adenylic acid, with an IC_{50} of approximately 10 μg/mL. The assay was insensitive to DMSO at concentrations up to 2%. Thus we have developed an oligonucleotide capture assay for the RNA helicase of Hepatitis C virus for the screening of Phytera's proprietary library of ExPAND® plant cell culture and μMARINE™ marine microbial culture extracts.

Future Conferences

1999 - March 21–26, Jerusalem, Israel

2000 - April 15–21, Baltimore, Maryland, USA

2001 - Seattle, Washington, USA (Tentative)

AN INVITATION
The Twelfth International Conference on Antiviral Research
March 21-26, 1999, Jerusalem, Israel

Dear Friends and Colleagues,

On behalf of the Organizing Committee, you are cordially invited to participate in the 12th International Conference on Antiviral Research, in Jerusalem, Israel in 1999. The Organizing Committee together with the International Society for Antiviral Research (ISAR) are making every effort to ensure a scientifically interesting meeting and that your stay with us will be a pleasant one.

The purpose of the International Conference on Antiviral Research is to provide an interdisciplinary forum at which investigators involved in basic, applied, and clinical research worldwide can meet to review recent developments in all areas of antiviral research. Specific topics to be covered in the program include synthesis and chemistry, biochemistry and mechanism of action, molecular biology and targeting, in vitro evaluations, animal models, pharmacokinetics, toxicology, and clinical trials. Within these areas of interest there will be invited "overview" speakers, oral presentations, and poster presentations.

The Conference will take place in the unique setting of Jerusalem - spiritual center of three great religions and a city of magnificent sites. We feel sure that Jerusalem will provide the appropriate milieu for a stimulating meeting.

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Jerusalem is... Archaeology and art, sacred sites and superb shopping, panoramic sun-swept promenades and moonlit concerts under the stars, classic dining, and fast-food restaurants, daytime walking tours and night club entertainment. Jerusalem is all this and more.

Information on the Scientific Program and instructions for submitting abstracts, housing and travel arrangements, and Conference registration will be included in the Second Announcement, to be sent to all members of ISAR in the summer of 1998. If you are not currently a member of the organization and wish to receive this information, please contact either of the Secretariats or refer to our web site at www.isar-icar.com.

We look forward to your participation.

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