diverse types of prodrugs was carried out including alkoxyalkyl (hexadecyloxypropyl, octadecyloxyethyl, hexadecyloxyethyl), pivaloyloxymethyl (POM), 2,2,2-(trifluoro)ethyl,2-butylsalicylyl esters as well as peptide-conjugated phosphonates. New synthetic procedures including the utilization of hexafluorophosphate coupling agents for esterification of the phosphonate function were developed. All HPMPDAP and cHPMDAP prodrugs were synthesized as phosphonate monoesters. A detailed anti-poxvirus and other antiviral testing as well as comparison of properties of single types of prodrugs was carried out. Alkoxyalkyl esters emerged as the most potent anti-poxvirus prodrugs of HPMPDAP and its cyclic form.

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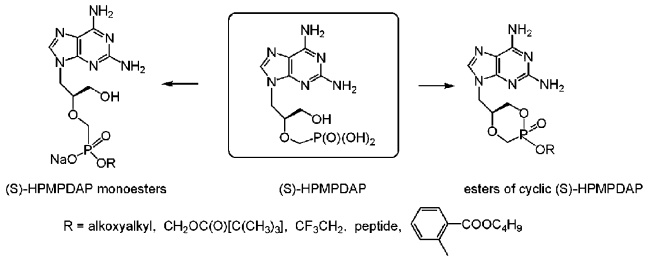

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New Nucleoside and *bis*-Nucleoside-Phosphonate Conjugates: Design, Stability, and Anti-HIV Evaluation

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Design of depot forms of anti-HIV drugs are widely used to reduce toxicity, improve drug pharmacokinetics, and overcome drug resistance. The obvious design rational for depot forms is that the conjugate will be converted by hydrolysis or enzyme action to active agents upon penetration into cells. Herein, we report the design, stability, and anti-HIV properties of phosphonate derivatives containing AZT, 3TC, and *bis*-nucleosides composed of mono- and heteronucleoside analogs with general structure Nu–O–P(O)(R)–O–Nu (Nu = AZT or 3TC). Among phosphonate depot forms of AZT or 3TC, the most perspective were their aminocarbonyl derivatives. They were stable in blood serum, displayed good anti-HIV activity and low toxicity in cell culture, improved pharmacokinetics, lower acute toxicity, and absence of cumulative effect if compared to that of AZT or 3TC, respectively. The stability in blood serum, anti-HIV activity and toxicity of phosphonate derivatives of *bis*-nucleosides were dependent on the structure of phosphonate moiety. Stability of morpholinecarbonyl-*bis*-AZT in blood serum was 30 min, at the same time for heterodimer ($R = ClCH_2$, Nu = AZT, 3TC) and homodimer ($R = CH_3–(CH_2)_5–NHC(O)$, Nu = AZT) was $\gg$6 h. The majority of compounds were less potent than parent nucleosides but their toxicity (CD_{50}) was considerably lower than those of the appropriate nucleoside. Therefore, a higher CD_{50} allowed better selectivity indexes (SI). Pharmacokinetic parameters of some phosphonate derivatives of nucleoside analogs and that of *bis*-nucleosides will be reported.

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RNA Polymerase Fidelity Variants of the Picornaviruses Uncover A Novel, Indirect RNA Mutagenic Activity for Amiloride Compounds

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Our laboratory studies the effects of RNA mutagens, and base analogs, ribavirin, 5-azacytidine and 5-fluorouracil on picornaviruses (poliovirus, Coxsackie B virus). In a screen to isolate RNA mutagen resistant variants of Coxsackie B3 virus, we identified a mutant presenting an A372V change in the viral RNA dependent RNA polymerase that conferred resistance to all 3 base analogs. This resistance was found to result from an increased polymerase fidelity, similar to our recently published work on ribavirin-resistant polioviruses that mapped to a different region of the polymerase (Vignuzzi et al., 2008). Interestingly, the A372V mutant had been previously isolated in a screen for resistance to amiloride compounds (inhibitors of Na^+ ion channels and the Na^+/H^+ exchanger), along with another polymerase mutant, S299T (Harrison et al., 2008). Since the same mutation would not expectedly confer resistance to two different antiviral mechanisms, we hypothesized that amiloride compounds had a previously unknown mutagenic activity. Indeed, we find that amiloride treatment of both Coxsackie virus and poliovirus increases their mutation frequencies. Furthermore, we show that higher fidelity variants of both viruses, presenting lower basal mutation frequencies, are more resistant to the RNA mutagenic effects of these compounds. Our results suggest that in addition to being replication inhibitors (as observed by other groups), amiloride compounds are the first described, non-nucleoside, indirect RNA mutagens. We are currently determining whether this mutagenic activity is the result of a direct interaction with the polymerase, or the result of intracellular alterations stemming from the inhibition of ion channels. Our data raises the question of whether this mutagenic activity is strong enough to act as an antiviral (through lethal mutagenesis) or whether it promotes viral evolution (through moderate mutagenesis). Implications for future drug development will be discussed.

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Overlap in Virus Specificity Leads to the Discovery of Small Molecules Active Against Rabies Virus, Cytomegalovirus, and Monkey Pox Virus

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The Prosetta Platform for cell-free protein synthesis (CFPS) and assembly of viral capsid-like structures has been used successfully to identify novel compounds with antiviral activity (see other

abstracts submitted). While a diverse group of pharmacophores have shown viral family selectivity, several chemical classes have shown activity across multiple viral families. Conversely, some hits identified early in the screening process as active against multiple viral families have progressed into subseries with greater selectivity and increased potency. We hypothesized that key recognition features necessary for antiviral activity in cell culture live virus assays were present in a good number of our early hits and likely targeted host factors. Thus it might be possible to bypass the need for plate screening to identify initial active small molecules for additional viral families. We tested this hypothesis on members of two unrelated viral families: rabies virus (RABV), a member of the Rhabodviridae (a single strand negative polarity RNA virus with a bullet-shaped capsid assembled in the cytoplasm) and Monkey pox (MPXV), a member of the Poxviridae (a double stranded DNA virus whose capsid forms in the cytoplasm). A subset of Prosetta's active antiviral pharmacophores were screened for effects on capsid assembly as measured by velocity sedimentation. Several hits were achieved for each of these viral families, and a significant number were found to be active against live virus in cell culture in each case. ELISA-based plate screens were established for RABV and MPXV and successfully validated the same hits. These findings demonstrate the versatility of CFPS-based drug screening, extend its reach to two more viral families, and suggest a novel strategy for management of emerging or engineered (bioweapon) viral threats.

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Cell-Free Protein Synthesizing Systems As Tools for Discovery of Drugs Inhibiting Viral Capsid Assembly

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Cell-free protein synthesis (CFPS) successfully reconstitutes transient protein–protein interactions difficult to detect in the crowded environment of living cells further complicated by downstream events (e.g. post-translational modifications and metabolism). Thus, CFPS allows a select subset of events to be studied in a more biochemically tractable manner. Upon CFPS programmed by mRNA encoding capsid protein(s), structures resembling viral capsids are formed as corroborated by biochemical, biophysical and electron microscopic analysis, first for Hepatitis B virus, and subsequently for HIV and Hepatitis C virus. We have extended the approach to 17 of the 23 families of viruses causing human disease. In each case, the capsid protein(s) assemble into high molecular weight structures via viral family-distinctive pathways. In some cases, subcellular fractions derived from mammalian tissues drive capsid protein-associated assembly events. These features can be accentuated by manipulation of conditions under which synthesis vs. assembly are conducted, for some viral families. Eight of these viral family-specific pathways have been converted into moderate throughput drug screens in a 384 well ELISA format, and have been used to screen all or part of a library of over 80,000 compounds conforming to Lipinski's rules. Multiple distinct chemical classes or pharmacophores MW < 500 Da have been identified that block putative steps of host-catalyzed capsid-related complex formation. Activity of many of these compounds have been demonstrated against live virus in cell culture for 10 viral families (see other abstracts being presented). In several cases hits have been successfully advanced to pre-lead status with enhanced potency and moderation of toxicity, and are being used for target identification and dissection of mechanism. In conclusion, an important new anti-viral drug discovery platform has been established and validated.

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A Chemoenzymatic Synthesis of Carbocyclic Nucleosides and Nucleotides

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In the last decades carbocyclic nucleosides attracted much attention due to their interesting antiviral activity. For example abacavir and carbovir were found to be potent inhibitors of the HIV reverse transcriptase. Entecavir was shown to be an anti-HBV agent, but also possesses activity against HIV, HSV-1, VZV and Influenza. To be antivirally active the nucleosides need to be phosphorylated intracellularly into their triphosphate metabolites in order to be incorporated into the growing DNA strand. Advantages of the carbocyclic compounds are higher stability against enzymatic degradation due to the replaced oxygen atom. Therefore, they are more flexible which should allow a higher rate of phosphorylation in contrast to natural nucleosides.

Here, we present a new and efficient chemoenzymatic synthetic strategy to carbocyclic nucleosides. Starting from cyclopentadiene it is possible to synthesize racemic 3-benzyloxymethylcyclopent-3-enol which can be used in a chemoenzymatic resolution using Pancreatin. The enantiomerically pure cyclopentenol precursors were converted into different 3′,4′-cyclopentenyl nucleosides by a modified Mitsunobu reaction (Ludek and Meier, 2005). In addition, the modification of the 3′,4′-double bond of the carbocyclic moiety can lead to further promising carbocyclic nucleosides (Reichardt et al., 2006). Furthermore, several carbocyclic analogues were converted into their mono-, di- and triphosphates. This was achieved by a nucleophilic attack of water or phosphate salts to the corresponding *cyclo*Sal-phosphate triester (Warnecke and Meier, 2009). Antiviral data of nucleoside analogues and phosphate triesters will be presented.

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