## 147

### Efficacy of 5-Halogenated 2′-Deoxyuridines on Vaccinia Virus Thymidine Kinase Positive and Negative Strains, and Influence of Cell Type on Antiviral Potency

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Thymidine kinase (TK) from orthopoxviruses plays an important role in the antiviral activity of certain thymidine analogs (Antiviral Res. 2006;71:1–6), since TK+ virus was more potently inhibited than TK− virus. To investigate this further we evaluated TK+ (wild-type) and TK− forms of vaccinia (WR strain) virus for inhibition by 5-fluoro (5-F), 5-chloro (5-Cl), 5-bromo (5-Br), and 5-iodo (5-I) derivatives of 2′-deoxyuridine (dUrd). 5-F-dUrd was non-selective in its action, inhibiting both TK+ and TK− viruses equally well (EC50's 0.01–0.03 μM), and exhibited considerable toxicity to rapidly dividing uninfected cells (IC50's 0.02–0.08 μM). The other compounds were nearly equal in antiviral potency, and were not toxic to cells at 1000 μM. They preferentially inhibited TK+ virus (EC50's 2–4 μM) over TK− virus (EC50's 55–90 μM) in monkey kidney cells. However, in mouse cells both TK+ and TK− viruses were inhibited at nearly the same concentrations (1–2 μM). To help explain these effects, cells were labeled with [3H]5-Br-dUrd, [3H]5-I-dUrd, or [3H]thymidine for 12 h. SAX HPLC results indicated that the intracellular amount of nucleoside triphosphate (NTP) was greatly increased in monkey kidney cells infected with TK+ virus compared to TK− virus. But similar amounts of NTP were produced in mouse cells infected with these viruses. These results explain why TK+ virus was more potently inhibited than TK− virus in monkey cells but not in mouse cells. Thus, the contributory role of viral TK to antiviral activity of thymidine analogs depends upon cell type.

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## 148

### Nucleosidic Fusion Inhibitors

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The chemistry, safety and pharmacology of nucleosidic drugs are all well-understood, as are their antiviral mechanisms. Whereas they target viral DNA replication, non-nucleosidic drugs target functions such as fusion. Fusion involves binding and conformational changes to bring virion envelopes and cell membranes into close apposition. Proper amphipathic compounds may be able to inhibit fusion by targeting the virion envelope to prevent such apposition. We screened nucleosides 5-derivatized to aryl-ethynyl/-propargylethers. Treated herpes simplex virus (HSV) virions were adsorbed onto cells and washed. The propargylether linker enhanced inhibition of infectivity (3-fold), as did replacing the sugar, or increasing the lypophilicity of the 5-aryl (2000-fold). The most potent compound, dUY11, had an $IC_{50}$ of 20 or 49 nM (HSV-1 or -2), was not cytotoxic (<4% non-viable cells; SI > 1000), and just marginally cytostatic at 70 μM (3500x$IC_{50}$).

dUY11 did not inhibit HSV DNA replication, gene expression or virion release. HSV virions treated with 7 μM dUY11 (350x$IC_{50}$) were adsorbed onto cells at 4 °C and washed. dUY11 had no effect on binding (7% bound in dUY11 or no drug; 0.5% in heparin). Expression of fluorescent reporters driven by HSV-inducible promoters indicates entry. There was no fluorescence when virions were treated with 2 μM dUY11. The only function between binding and entry is fusion between two lipid bilayers, virion envelopes and cell membranes. Whereas envelopes are mostly for fusion, cell membranes are selective barriers; their lipids, proteins, curvatures and fluidities also differ. Untreated virions were adsorbed onto treated cells, or treated virions onto untreated cells. Virion treatment blocked infectivity ($IC_{50}$ 20 nM), whereas that of cells only reduced it by 75% ($IC_{50}$ 5.4 μM). dUY11 inhibited infectivity of vesicular stomatitis or Sindbis virus ($IC_{50}$ 6 or 58 nM, respectively), which bind to unrelated receptors to fuse to endosomes. dUY11 targets are thus conserved among otherwise unrelated enveloped viruses.

Amphipathic nucleoside derivatives inhibit entry by preventing fusion between virion and cell membranes, providing novel scaffolds for broad-spectrum fusion inhibitors.

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## 149

### Enhanced Potency and Efficacy of 29-mer shRNAs in Inhibition of Enterovirus 71

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Enterovirus 71 (EV71) is the main causative agent of hand, foot, and mouth disease (HFMD) in young children. It has been associated with severe neurological complications and has caused significant mortalities in large-scale outbreaks in Asia. In this study, we demonstrated an enhanced silencing of EV71 through the use of chemically synthesized 29-mer shRNAs. The 29-mer shRNAs were designed to target three highly conserved regions of EV71 genome. Transfection of rhabdomyosarcoma (RD) cells with the 29-mer shRNAs significantly inhibited EV71 replication in a dose dependent manner as demonstrated by reduction of viral RNA, VP1 protein and plaque forming units. The inhibitory effects were more potent and were achieved at 10-fold lower concentrations when compared to 19-mer siRNAs reported previously (Sim et al., 2005). The viral inhibitory effects lasted

72 h post-infection and there was no adverse off-target silencing effect. Gene silencing by 29-mer shRNAs targeted at the $3D^{pol}$ region (sh-3D) was the most effective, achieving 91% viral inhibition. Further evaluation found that no enhanced inhibitory effects were observed when sh-3D was cotransfected with each of the other two candidates. This study showed an improvement in triggering RNAi using the more potent 29-mer shRNAs, indicating its therapeutic potential against EV71.

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## 150

## Crosstalk Between Scavenger Receptors (SR-A) and Toll-like Receptors (TLR) Results in Rapid Pro-Inflammatory Cytokine Differentiation in Monocytes Exposed to Cytomegalovirus (CMV)

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**Introduction:** Disease from CMV occurs rarely in immunocompetent hosts. Monocytes are an important first defense against CMV disease due to multiple pattern recognition receptors including SR-A and TLRs. However, SR-A and TLR receptor interactions that contribute to pro-inflammatory cytokine induction immediately after monocyte exposure to HCMV are not defined. A better understanding of cellular mechanisms and signaling cascades during innate immune and pro-inflammatory responses will be important in studying CMV pathogenesis. To test this hypothesis, we used the human monocytic THP-1 cell line to investigate the interacting roles of these receptors shortly after contact with CMV and their involvement in inducing pro-inflammatory cytokines.

**Methods:** THP-1 cells were incubated for 1, 5 and 10 min with low passage wild-type CMV isolated from a congenitally infected infant. After incubation, total RNA was extracted from cells and treated with DNase. RNA was subjected to reverse transcription. cDNA was analyzed by semi-quantitative PCR for gene expression.

**Results:** At 1 min post CMV exposure, mRNA levels were elevated for SR-A and Lyn, an SR-A associated cytoplasmic tyrosine kinase. This suggests SR-A and Lyn are activated simultaneously immediately after THP-1 exposure to CMV. Surprisingly, at 10 min, there was a dramatic elevation of TLR2, implying SR-A is acting upstream of TLR2. However, mRNA levels of TLR3 and TLR9 were inhibited 1, 5 and 10 min after THP-1 exposure to CMV. Marked elevation of TNF-alpha over baseline at 10 min suggests that SR-A and TLR crosstalk plays a role in TNF-alpha activation. Similar elevation was seen for IL-12 while IFN-beta levels were unaffected, suggesting IFN-beta is not responsible for earliest inflammatory responses in CMV-exposed THP-1.

**Conclusion:** Our findings establish for the first time a novel paradigm in CMV-exposed monocytes between SR-A and TLR receptor signaling pathways that are important for induction of pro-inflammatory cytokine expression. Such a relationship in CMV-exposed monocytes between these two receptor families that induce pro-inflammatory cytokines could be important in CMV infection outcome.

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## 151

## Solid-phase synthesis of the anti-HBV dinucleotide SB 9000—Microwave-assisted Functionalization of Solid Supports

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SB 9000 is being developed as a new class of antiviral agent against HBV. For the in vitro and in vivo studies of SB 9000, we needed access to large amounts of SB 9000, which is most conveniently prepared by solid-phase synthesis using phosphoramidite chemistry.

For the large-scale manufacture of SB 9000, availability of nucleoside-loaded solid support is critical. Recently, we have developed ultra fast methods for functionalization of controlled-pore glass (CPG) using microwave-assisted procedures. This technology provides rapid access to amino-functionalized and succinylated-CPG in large-scale. Thus, following a 10-minute, microwave exposure of a slurry of native CPG in aminopropyl-(triethoxy)silane, amino-propyl CPG was obtained. Further reaction of the amino-support with succinic anhydride in the presence of coupling reagent and catalyst, under microwave conditions for 5 min, resulted in succinylated CPG. These methods were applicable to a range of supports including LCAA-CPG, Primer support®, and so on. Concurrently, we have also developed efficient procedures for the loading of nucleosides on functionalized CPG using LOTUS®, a versatile multi-purpose chemical synthesis reactor. We have obtained loadings ranging from 80 to 220 μmol/g depending upon the solid support used.

5′-O-dimethoxytrityl-2′-deoxy-adenosine-loaded CPG, obtained following these protocols, was used for the synthesis of SB-9000. The sequence of synthetic steps involving detritylation, coupling, sulfurization, were all carried out in an inert atmosphere using LOTUS® reactor. Following the synthesis, cleavage and removal of protecting groups was achieved by treatment with 28% NH4OH to give crude SB 9000. HPLC purification, followed by desalting, endotoxin-removal, and lyophilization gave SB 9000 in >95% purity. The Rp, Sp-SB 9000 was fully characterized by spectral methods, and was employed for all in vitro and in vivo studies.

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