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Toward Orthopoxvirus Countermeasures: A Novel Heteromorphic Nucleoside of Unusual Structure

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Abstract

Two privileged drug scaffolds have been hybridized to create the novel heteromorphic nucleoside 5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4*H*-chromen-4-yl)-1-(2-deoxypentofuranosyl)pyrimidine-2,4-(1*H*,3*H*)-dione (**2**). Compound **2** inhibited the replication of two orthopoxviruses, vaccinia virus (VV) (EC_{50} = 4.6 ± 2.0 μM), and cowpox virus (CV) (EC_{50} = 2.0 ± 0.3 μM). Compound **2** exhibited reduced activity against a thymidine kinase (TK) negative strain of CV, implying a requirement for 5′-monophosphorylation for antiorthopoxvirus activity. Compound **2** was efficiently phosphorylated by VV TK, establishing that VV TK is more promiscuous than previously believed.

Smallpox, although declared eradicated as a natural disease in 1983 by the World Health Organization, now stands as the most potentially devastating of all bioterrorist threats.[1,2] It is presently the policy of the U.S. Government to provide two FDA-approved drugs for the treatment of smallpox and to have two others in the pipeline, ideally with different modes of action.[3] One drug, cidofovir (Vistide), licensed to treat cytomegalovirus (CMV) retinitis in HIV-infected patients, is available through a special protocol (Investigational New Drug, IND) for emergency treatment of smallpox or vaccine reactions (http://www.bt.cdc.gov/agent/smallpox/vaccination/cidofovir.asp) if vaccinia immune globulin (VIG, in limited supply) is not effective.[4,5] Progress has been made on development of oral dosage forms of cidofovir,[6–10] but these are not yet available in the clinic. Some agents for the treatment of orthopoxvirus infections are in preclinical or clinical development. These include inhibitors of viral morphogenesis (TTP-6171)[11] and viral release (ST-246)[12] as well as cellular (i.e., Erb-1 kinase inhibitors, CI-1033)[13,14] and tyrosine kinase inhibitors (Gleveec, STI-571).[15] Nonetheless, there presently is no drug approved by the FDA to treat smallpox.

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Supporting Information Available: Synthesis, characterization, and HPLC purity of new compounds reported. This material is available free of charge via the Internet at http://pubs.acs.org.

We have pursued a chemistry-driven strategy for the discovery of lead molecules with anti-orthopoxvirus activity.[16,17] Our approach to new orthopoxvirus antivirals has been guided by the following considerations: (a) since the "privileged"[18,19] structure of nucleosides has led to a variety of efficacious antiviral agents,[20] the nucleoside scaffold is an excellent point of departure in the search for new antiviral drugs; (b) other privileged[18,19] molecular scaffolds exist that have spawned a significant number of drugs and other biologically active agents, and these also can be used to discover molecular "masterkeys";[21] (c) 5-formyl-2′-deoxyuridine is a neglected but powerful synthon for the generation of novel nucleoside structures that can be employed in multicomponent reactions[22–25] (MCR) to generate chemical diversity.

In this study, a modified benzofuran–nucleoside chimera was generated in a MCR originating with 5-formyl-2′-deoxyuridine.[26–28] Benzofuran congeners form the nucleus of many biological active molecules.[29–32] Singh et al.[33] gained entry to these fused pyrans by reactions of 1,3-oxazinanes and oxazolidines with various carbon nucleophiles. We adapted this to the reaction of 5-formyl-2′-deoxyuridine with malononitrile and 1,3-cyclohexanedione to obtain a novel nucleoside. The synthesis was carried out using 5-formyl-2′-deoxyuridine[26–28] in a multicomponent reaction with malononitrile and 1,3-cyclohexanedione in EtOH to give 5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4*H*-chromen-4-yl)-1-(2-deoxypentofuranosyl)pyrimidine-2,4(1*H*,3*H*)-dione (**2**) (Scheme 1). Compound **2** was obtained as a 1:1 diastereomeric mixture arising from the generation of a chiral carbon at position 4 of the chromone ring.

The antiviral activities of **2** (Table 1) were determined in human foreskin fibroblast cells, and the challenge orthopoxviruses were vaccinia virus (VV) or cowpox virus (CV). An initial evaluation was performed using the viral cytopathogenic effect as the endpoint. A second confirmatory assay involved plaque reduction. The concentration of agent that inhibited viral CPE or plaque formation by 50% was defined as the EC_{50}. The effect of the potential antiviral agent on uninfected host cell viability was ascertained by Neutral Red uptake as a measure of cellular cytotoxicity. The concentration that reduced Neutral Red uptake by 50% was defined as the CC_{50}. Compound **2** had no significant cytopathic effect on uninfected cells under these conditions (CC_{50} > 300 μM).

Compound **2** was also evaluated against a thymidine kinase (TK) deficient strain (TK:GFP lacZ) of CV. CDV does not require phosphorylation to be active because it is a monophosphate analogue.[4,5,39,40] Therefore, its activity is quite similar in TK^+ and TK^- virus strains. 5-Iodo-2′-deoxyuridine (idoxuridine) is known to be activated by the viral TK[41] such that it is much less effective against TK^- viruses.

The data of Table 1 clearly show that **2** is active only against the TK^+ strain of CV, suggesting a specific 5′-monophosphorylation of this compound by the virus enzyme. That **2** indeed is a substrate for VV TK was confirmed by in vitro assays with recombinant VV TK. Under conditions wherein thymidine itself possessed a K_m of 49 ± 7.6 μM and a V_{max} of 289 ± 137 μmol min^{-1} mg^{-1}, **2** was found to have a K_m of 43 ± 1.4 μM and a V_{max} of 77 ± 5 μmol min^{-1} mg^{-1}. Thus, **2** is a good substrate and is efficiently phosphorylated by the enzyme.

These results have several important consequences for orthopoxvirus antiviral discovery and development. First, the requirement for the poxvirus TK for antiviral activity attests that **2** can be expected (as so far suggested by the cell culture studies of Table 1) to be of minimal toxicity to uninfected cells. Second, these foregoing data also imply that the orthopoxvirus TK (as embodied by the VV and CV genomes) may not exhibit the extremely limited substrate specificity characteristic of other type II highly discriminating TKs. VV TK originally was classified as a type II TK because of its substrate specificity, sequence homology to other type II kinases, and tetrameric configuration.[42–46] To date, the only published recognized substrates for VV TK are thymidine, 2′-deoxyuridine, and 5-bromo-2′-deoxyuridine. The data reported here with **2** signify that, as for the herpes virus TKs, orthopoxvirus TKs are more promiscuous kinases than the cellular homologues, thereby providing fertile terrain for more diverse structure interrogation for candidate antiorthopoxvirus agents. Third, the unique structure of **2** suggests the possibility of a novel mode of action. Last, the recruitment of the versatile 5-formyl-2′-deoxyuridine and the adoption of the multicomponent reaction strategy provide access to an uncharted domain of structural diversity for exploration in antiviral drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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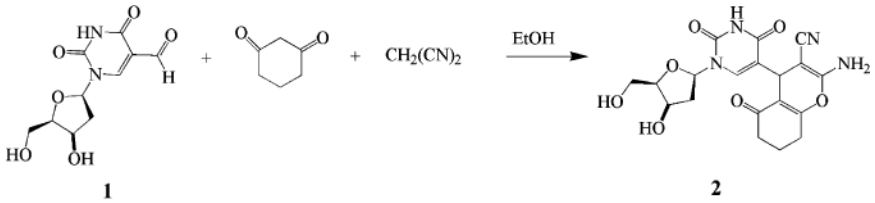

Scheme 1.
Synthesis of Compound **2**

Table 1

Antiorthopoxvirus Activities[a]

	efficacy (EC_{50},[c] μM)					
compd	**VV[b] CPE**	**VV[b] PR**	**CPV[d] TK⁺ lacZ**	**CPV[b] PR**	**CPV[d] TK⁻ lacZ**	**toxicity (CC_{50},[e] μM) Neutral Red uptake**
cidofovir	3.2	24 ± 12	3.3 ± 1.1	40 ± 6.1	5.2 ± 3.9	>317 ± 0
2	0.6	4.6 ± 2.0	0.8 ± 0.1	2.0 ± 0.3	28 ± 2.7	>300 ± 0
5-iodo-2′-deoxyuridine		6.0 ± 0.2	0.4 ± 0.1	2.0 ± 0.2	27 ± 3	>260[a]

[a] Procedures adapted from Kern et al.[34] Assays were performed according to the procedures described previously[35–37] for activity against VV and CV and for cytotoxicity (Neutral Red uptake assay) in human foreskin fibroblast (HFF) cells. Briefly, to determine efficacy, initial cytopathogenic effect (CPE) assays were performed in 96-well plates seeded with HFF cells. Varying concentrations of drug were added to monolayers of HFF cells and challenged with VV or CV at 1000 PFU per well (incubation at 37 °C for 7 days). Confirmatory assays involving plaque reduction (PR) assays were performed using HFF cells seeded in six-well plates 2 days prior to use and infected with VV or CV by the addition of 20–30 PFU per well. Plates were incubated for 1 h. Various concentrations of drug were then added to triplicate wells, and plates were incubated at 37 °C for 3 days. Toxicity was evaluated using uninfected HFF cells seeded in 96-well plates incubated with various concentrations of drug for 7 days at 37 °C.

[b] Virus used for challenge: VV (Copenhagen) or CV (Brighton).

[c] Values are the mean (standard deviation of two or more assays.

[d] CV strains δcrmA (TK^+) and TK:GFP lacZ (TK^-) were obtained from Pete Turner (University of Florida, Gainesville, FL) and were described previously.[38] Values were obtained using a β-galactosidase assay to determine antiviral activity.

[e] CC_{50}: concentration that causes a cytotoxic effect (as ascertained by Neutral Red uptake) on 50% of uninfected cells.