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Discovery of a novel series of inhibitors of human cytomegalovirus primase

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Abstract—Infection by human cytomegalovirus (hCMV) remains a potent threat to susceptible people throughout the world. We have discovered a series of imidazolyl-pyrimidine compounds, which were found to be irreversible inhibitors of the hCMV UL70 primase based on results from radiolabeling and SAR studies. Two promising analogs are described that rival ganciclovir and cidofovir in antiviral potency and possess improved cytotoxicity profiles.

In spite of advances in anti-hCMV therapy and the introduction of (HAART),[1] hCMV infection remains a significant health problem.[2] Cytomegalovirus (CMV) is a common virus of the herpes family with estimated infection rates of 50–85% of the United States population. In the countries of Asia, Africa, and South America the incidence is upwards of 95%. Generally the virus is dormant but in immuno-compromised patients, such as HIV carriers, the infection rate can be as high as 90–95%, with hCMV disease manifesting itself in 30–40% of those cases. Patients undergoing transplants often develop hCMV disease that may lead to graft rejection, organ dysfunction, and, in the case of bone marrow or heart–lung transplantation, interstitial pneumonia.[3] Additionally, hCMV is the most common form of congenital infection, with 40,000 infants affected yearly in the US alone; 10% of these will develop CMV disease with symptoms such as microcephaly and mental retardation.[4] Current therapy is hampered by the difficulties associated with drug delivery, drug-resistant hCMV strains,[5] and pronounced side effects. Most therapeutic agents are administered intravenously or have poor oral bioavailability.[6] Side effects include neutropenia, thrombocytopenia, and nephrotoxicity.[7]

In the course of high-throughput screening against hCMV using an hCMV-luciferase replication assay[8] we discovered the imidazolyl-pyrimidine **1**. Medicinal chemistry was initiated around the core of pyrimidine **1**, varying the substituents at the 2-, 4-, 5-, and 6-positions in an effort to improve the potency and the physico-chemical properties of the molecule.[9] Figure 1 summarizes important aspects of the SAR results. The anti-hCMV activity (IC_{50}) of **1** was found to be 0.3 and 0.55 μM in the viral DNA replication (dot blot) and luciferase reporter cell-based assays, respectively.[10] In general, the potency of compounds in these two

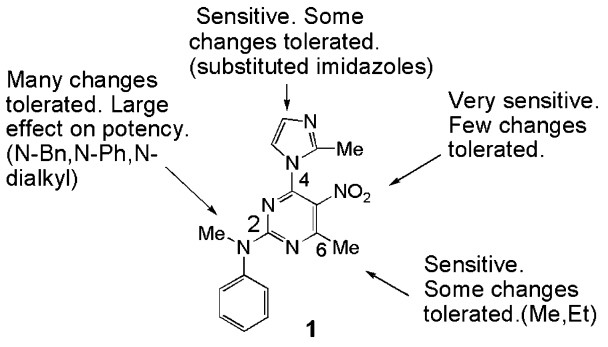

Figure 1. Summary of the early SAR.

Keywords: Cytomegalovirus; CMV; Ganciclovir; Cidofovir; Imidazolyl-pyrimidine; UL70 primase; Irreversible inhibitor; Radiolabeling; Tritiation.
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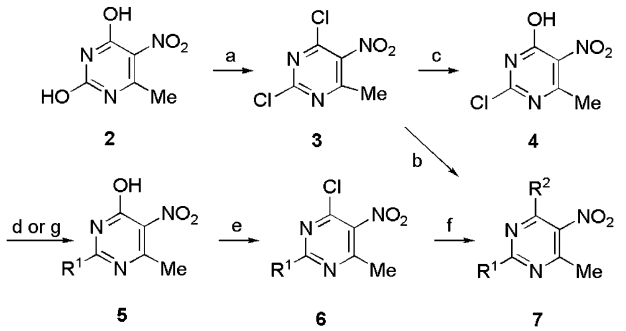
assays was comparable, although the dot blot assay was used for the initial evaluation of all compounds. In addition, the cytotoxicity of all compounds was tested against two different cell types (Jurkat cells and the human foreskin fibroblasts (HFF) utilized for viral infection).

The synthesis of the majority of the analogs is outlined in Scheme 1. 2,4-Dichloro-6-methyl-5-nitropyrimidine (**3**) was prepared in a modification of the known procedure.[11] We found that the yield of **3** could be enhanced by the use of pre-dried tetraethyl ammonium chloride and anhydrous acetonitrile. Compound **3** was hydrolyzed selectively with sodium acetate in acetic acid to provide compound **4**,[12] which on treatment with various amine or alcohol nucleophiles, followed by $POCl_3$, provided an intermediate chloro adduct **6**. This material was then subjected to a refluxing solution of the desired imidazole to give the final product **7**, usually in good overall yield. Alternatively, the 2,4-dichloropyrimidine **3** was subjected to a controlled amount of an amine and imidazole under refluxing conditions, to provide a complex mixture of three main isomeric products, in which the desired material was usually the minor component.

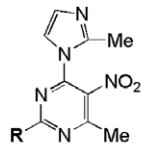
Using the chemistry outlined in Scheme 1, a small library of compounds was constructed in order to evaluate the lead and optimize the contribution of the 2-anilino group. Some of these SAR results are shown in Table 1. Most analogs tested were worse or similar in potency to **1** except for benzyl amines such as the *N*-methylbenzyl amine **9** and the indane-containing analog **12.** They inhibited viral replication (dot blot assay) with IC_{50} values of 0.06 μM and 0.05 μM, respectively. Additionally, **9** and **12** were found to inhibit viral growth, as measured by a one-step viral yield assay, with $IC_{99.9}$ value of 0.2 μM and 0.1 μM, respectively.[13] Viral plaque formation was also completely inhibited with $IC_{99.9}$ value of 0.5 μM.[14]

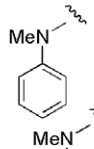
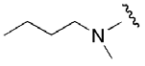
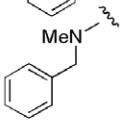
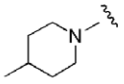
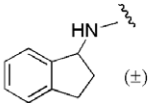
These two compounds showed a significant improvement in anti-CMV potency relative to the two marketed drugs cidofovir (CDV) and ganciclovir (GCV). Their



Scheme 1. Reagents and conditions: (a) Et_4NCl, dimethylaniline, $POCl_3$, CH_3CN, 87%; (b) 1–2 equiv 2-methylimidazole, 2–4 equiv amine, THF, −78 °C, 6–10%; (c) NaOAc, AcOH, EtOH, H_2O, 79%; (d) NaOAc, amine, EtOH, reflux (R^1 = 1-aminoindane 66%); (e) $POCl_3$, 100 °C (R^1 = 1-aminoindane 51%); (f) R^2 = various imidazoles, CH_3CN, reflux (R^1 = 1-aminoindane, R^2 = 2-methylimidazole, 95%); (g) NaOEt, EtOH, reflux, R^1 = OEt, 79%.

Table 1. Anti-hCMV activity of early analogs



Analog	R	Viral DNA IC_{50} (μM)	Analog	R	Viral DNA IC_{50} (μM)
1		0.3	10		0.3
9		0.061	11		0.3
8	OEt	0.67	12	 (±)	0.053

cellular toxicity profiles also compared favorably to those of GCV and CDV in Jurkat and HFF cells,[15] and in the more sensitive bone marrow cell assay (colony-forming unit of granulocyte/macrophage or CFU-GM) providing a large window between cellular toxicity and antiviral activity (Table 2).[16]

While **9** and **12** are more potent than the original compound **1**, and compare favorably to GCV and CDV in terms of antiviral activity and cytotoxicity, their overall physicochemical profiles are not optimal. They remain poorly soluble and are metabolically labile. For example, incubation of **9** with rat liver homogenates for 2 h in the presence of NADPH led to 63% degradation and formation of two adducts, one arising from the demethylation of the *N*-methyl-benzyl moiety and another arising from the hydroxylation of the phenyl ring. Subsequent SAR studies were carried out without the *N*-methyl group and with the oxidation sites blocked. The stability of the cyclic analog **12** was much greater than that of **9**. After 2 h incubation with rat liver homogenate, there was only a 10% loss of parent and a single phenolic metabolite was formed.

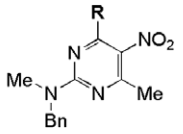
Notwithstanding the improvement of antiviral potency, a significant amount of our initial efforts were centered on the imidazole/nitro group dyad. It was postulated that the activity of these compounds might be the result of covalent bond formation between a nucleophile on an unknown viral protein and the pyrimidine ring by S_NAr displacement of the imidazole. Interestingly, most changes in the 4-position of the pyrimidine had large negative effects on potency, becoming more pronounced as the substituent deviated further from the imidazole core.

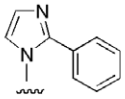
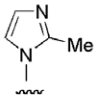
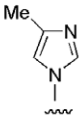
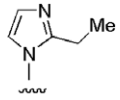
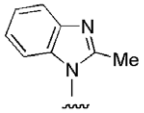
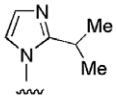
Table 3 contains analogs with various substituted imidazoles, showing that activity decreases as the size of the substituent at the 2′-position is increased. The 2′-phenyl imidazole analog **16** was 250 times weaker than the imidazole-containing compound **13**. Additionally, the structurally similar but only weakly electrophilic

Table 2. Antiviral activity, cellular toxicity and bone marrow cellular toxicity comparison of **1**, **9**, **12**, GCV (ganciclovir), and CDV (cidofovir)

Drug	In vitro activity ($IC_{99.9}$ (μM))		Cellular toxicity (IC_{50} (μM))		
	CMV yield	Plaque reduction	HFF toxicity	Jurkat toxicity	CFU-GM
1	2.0	2.5	100	80	30
9	0.2	0.5	>100	25	30
12	0.1	0.5	>100	100	40
GCV	5.0	10	>100	>100	30
CDV	1.0	2.5	>100	100	10

Table 3. Anti-CMV activity of imidazole analogs



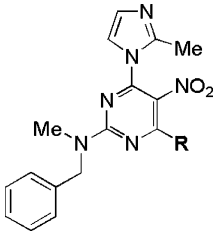
Analog	R	anti-hCMV (Luc.activity) IC_{50} (μM)	Analog	R	anti-hCMV (Luc.activity) IC_{50} (μM)
13		0.02	**16**		5.0
9		0.039	**17**		10
14		0.19	**18**		>30
15		0.77	**19**		>30

benzimidazole **18** and the unreactive pyrrole **19** were even less active. These results are consistent with the postulate that this series of analogs act as covalent modifiers of a target molecule, presumably a protein. In addition, there appears to be a steric limitation with respect to the recognition of these molecules by the viral target, as evidenced by the significantly decreased potency of compound **17**.

Compounds were synthesized in order to replace or remove the nitro group, to further assess the electrophilic requirements for this lead. Various alternate electron-withdrawing groups were selected but they all proved to be much weaker in potency, including nitrile **20** (Fig. 2).

An effort was then undertaken to explore the SAR around position 6 of the pyrimidine ring, in conjunction with a radiolabeling study (see below). It appeared that the methyl group was required for anti-CMV activity, as

Table 4. Anti-CMV activity of variously substituted pyrimidines



Analog	R	Anti-hCMV (Luc-activity) IC_{50} (μM)
24	H	3.0
9	Me	0.039
25	Et	0.17
26	*i*-Pr	3.0
27	Cl	>30
28		6.0

removal or elongation beyond an ethyl group led to a reduced effect on antiviral activity (Table 4).

Because the mode of action remained in doubt, a series of biological experiments were designed to determine if and where the inhibitors were covalently bound. Our synthetic approach employed alkylation of **9** with Eshenmocher's salt, followed by quaternization of the amine **28** and subsequent elimination to provide an alkene product **29**. This alkene was radiolabeled with tritium via a controlled hydrogenation, taking advantage of the more reactive primary alkene relative to the hindered and less reactive nitro moiety. This procedure provided a pure sample of tritiated (50 Ci/mmol) [^{3}H]$_2$-**25** after an HPLC purification to remove the byproducts of over-reduction[17] (Fig. 3).

The [^{3}H]$_2$-**25** was delivered to HFF cells infected with the rhCMVLuc wild type virus. A comparison of the protein content of infected and uninfected cells on polyacrylamide gel under denaturing conditions showed one unique band that was specific to the infected cells,

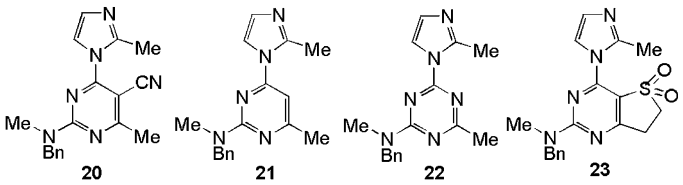


Figure 2. Analogs of **1** that lack the nitro group (all with IC_{50} > 30 μM).

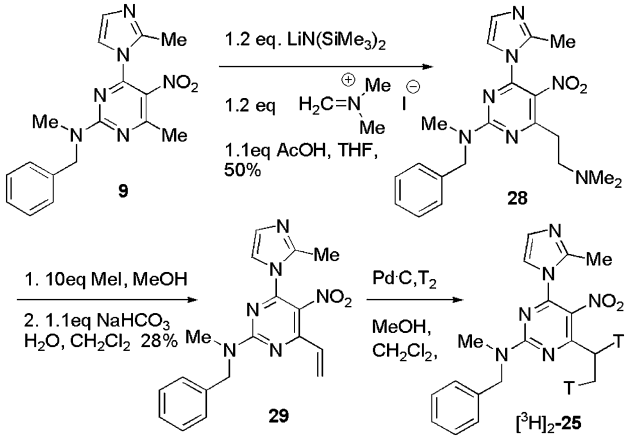


Figure 3. Synthesis of $[^3H]_2$-**25**.

appearing after 48 h postinfection (Fig. 4). From this and subsequent work, we determined that the protein band was part of the viral helicase:primase complex. Of the three proteins in this complex, UL102, UL105, and UL70, it was determined through immunoprecipitation experiments that the binding protein was the UL70 primase. A cartoon of this binding event is shown below (Fig. 5).[18]

Interestingly, the group of analogs described here are highly specific for hCMV. They have no effect on the replication of other viruses, such as HSV, EBV, HIV, HHV6, rat CMV, guinea pig CMV, or mouse CMV. Rhesus CMV is the only other CMV virus

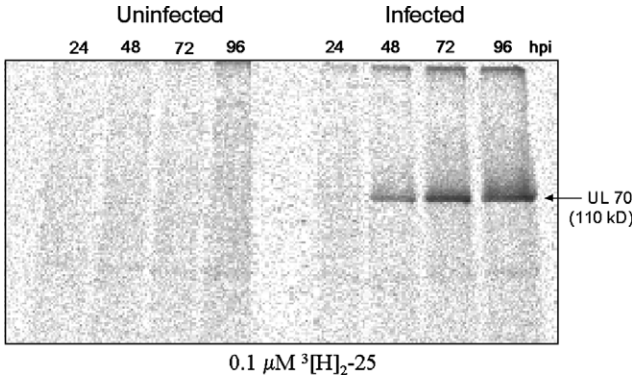


Figure 4. A phosphoimage of the SDS polyacrylamide gel showing the covalent linkage between a 110 kDa protein (UL70 primase) and 3$[H]_2$-**25**. hpi, hour postinfection.

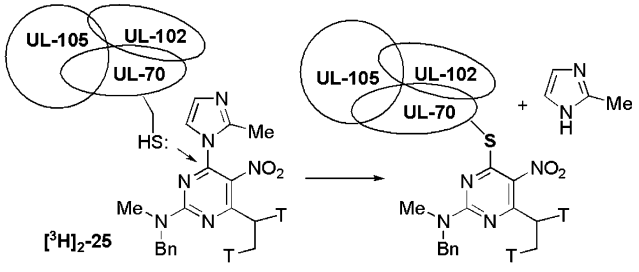


Figure 5. Nucleophilic attack of hCMV UL-70 on compound **25**.

tested that shows any sensitivity to these compounds, typically with at least 10-fold decreased potency compared to human CMV.

In summary, we have discovered a novel class of non-nucleoside hCMV inhibitors, which bind in an irreversible manner to hCMV UL70 primase. Two compounds, **9** and **12**, have been identified in this preliminary medicinal chemistry effort that possess optimized profiles of antiviral potency and a promising therapeutic window (antiviral activity vs cytotoxicity). Additional medicinal chemistry will be reported on our efforts to improve the overall PK profile of this series while retaining and enhancing their potency.

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8. The luciferase screen involved a recombinant virus (rhCMVLuc) containing a luciferase reporter gene, under the control of hCMV UL99 promoter, inserted in the CMV genome between US9 and US10. HFF cells (10^4 cells/well in 96-well plates, incubated for 2 h) were infected with rhCMVLuc, at a multiplicity of infection (MOI) of 1 plaque forming unit/cell (pfu/cell). After 1 h, the inoculum was removed and the test drugs (0.005–5 μM or 0.05–50 μM) in fresh media were added and incubated for an additional 48 h at 37 °C. Luciferase activity was measured with the addition of $Mg(OAc)_2$, 30 mM Tricine (pH 7.8), and 200 μM EDTA. The plates were read with a luminocount machine and the data quantified with Microsoft Excel. IC_{50} values represent the concentration of test compound required to inhibit hCMV DNA replication by 50%.

9. Some aspects of this work were presented at the 41st Interscience conference on Antimicrobial Agents and Chemotherapy, presentations 1672 and 1673. Chicago, IL, December 18th 2001 (posters).
10. The CMV DNA replication assay (dot blot): The HFF cells were handled exactly as in Ref. 8 except there were 2×10^4 cells/well, rhCMVLuc, clinical isolates, and wild type viruses were used, and the additional incubation time was 72 h not 48 h. After 72 h, the media were removed and lysis buffer (0.4 N NaOH, 0.5 M EDTA) was added. An aliquot was applied to a zeta-probe membrane by microfiltration, which was washed, dried (slightly), and subjected to UV radiation (Stratalinker) to crosslink the DNA to the membrane. The immobilized DNA was hybridized with a hCMV-sequence-specific radiolabeled probe, washed again, dried, and treated to phosphoimaging (plate and imager) to detect ^{32}P. The data were quantified with Mac BAS and MS Excel to provide IC_{50} values.
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13. The viral yield assay involves HFF cells treated exactly as reference 10 except wild type, clinical isolates or drug-resistant viruses were used and the incubation time was 5–7 days not 72 h. At this time when the cytopathic effect (CPE) was observed, the supernatant in each well was diluted (1:2) 16 times into fresh media, and used to infect fresh HFF cells (10^4 cells/well of 96-well plates). After 1 h, each inoculum was removed and fresh media were added to each well. The newly infected cells were incubated for 5–7 days when CPE was again apparent. The viral yield was quantified as before and the $IC_{99.9}$ determined to be the concentration of drug required to inhibit the hCMV replication by 99.9%.
14. The plaque reduction assay was performed by counting plaques from infected cells (10^5 cells with 1000 pfu of wild type, clinical isolates or resistant viruses) after a 10-day incubation. $IC_{99.9}$ values represent the amount of drug required to inhibit plaque formation by 99.9%.
15. The cytotoxicity assay in HFF or Jurkat cells was performed by seeding cells into 96-well plates, waiting 3 h, and then adding compound. This was followed by Alamar blue (10 μL) staining at various time points (0, 24, 48, and 72 h). After incubating an additional 3 h, the fluorescence was determined (PerSeptive Biosystems CytoFluor II) and quantified (MS Excel) providing an IC_{50} value, concentration of compound that inhibited cell growth by 50%.
16. The cytotoxicity assay in bone marrow cells was performed in the following way. Human bone marrow cells were harvested, washed [Iscoves modified Dulbecco's medium (IMDM)–2% FBS], and the viable nucleated or mononuclear cell number was determined. The cells were diluted with IMDM–2% FBS until there were 10^6 cells/mL at which point the drug was added. The cells were diluted 1:10 methocult (stem cell technologies), vortexed, and plated onto tissue culture dishes. After incubating at 37 °C in a humid 5% CO_2 atmosphere for 15 days, the CFU-GM colonies were quantified by microscopic inspection (inverted microscope). The concentration of drug that inhibited colony formation by 50% (IC_{50}) was calculated.
17. American Radiolabeled Chemicals Inc., St. Louis, MO, performed the final tritiation step.
18. Details of this and other experiments that characterize the exact cysteine residue on UL70 responsible for imidazole displacement will be published in due course.