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SAR studies on a novel series of human cytomegalovirus primase inhibitors

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Abstract—A novel series of imidazolylpyrimidines were found to possess inhibitory activity against the human CMV UL70 primase. Extensive SAR studies on an HTS lead led to potent, orally bioavailable compounds with anti-CMV IC_{50} values of 150 nM in both viral yield and viral DNA replication assays and with a much reduced cytotoxicity compared to marketed treatments ganciclovir and cidofovir.

Cytomegalovirus (CMV) is a subgroup of herpesviruses that is widely distributed in nature. Human cytomegalovirus (hCMV) infects, by age 40, about 50% of the population outside of urban centers and up to 80% of the population within the cities in North America.[1] CMV infection in normal hosts results in a sub-clinical response that is usually not diagnosed or treated. However, infection of allograft recipients (kidney, liver, heart, and bone marrow) is a major cause of graft rejection.[2–4] Human CMV also causes retinitis in AIDS patients with low CD4 counts, a situation expected to continue unless an appropriate treatment against hCMV is implemented. The existing treatments for hCMV suffer from poor oral bioavailability, sub-optimal antiviral activity, as well as propensity for induction of severe neutropenia.[5] Additionally, the emergence of ganciclovir- and cidofovir-resistant virus has resulted in an increase in the rate of disease relapse in the high-risk patient population.[6] High-throughput screening using a cell-based hCMV-luciferase replication assay discovered compound **1**.[7] This and related compounds were found to be inhibitors of the hCMV UL70 primase.

In an earlier publication,[8] we disclosed the initial SAR results and radiolabeling experiments that led to the

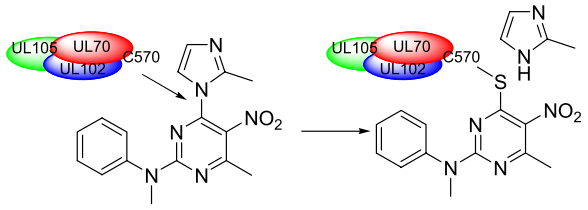

Figure 1. Proposed mechanism of action for compound **1** and analogs.

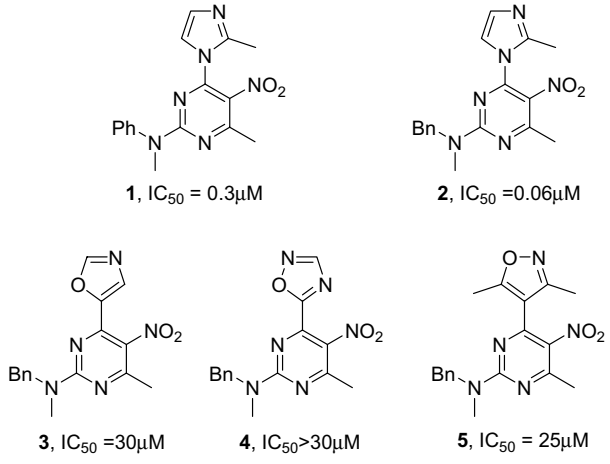

Figure 2. Attempts to prepare a non-covalent CMV primase inhibitor. (IC_{50} values shown refer to inhibition of viral DNA replication determined by Dot blot assay[9]).

Keywords: Anti-viral; hCMV; Primase; Cytomegalovirus; Inhibitor.
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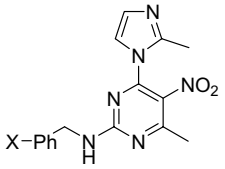
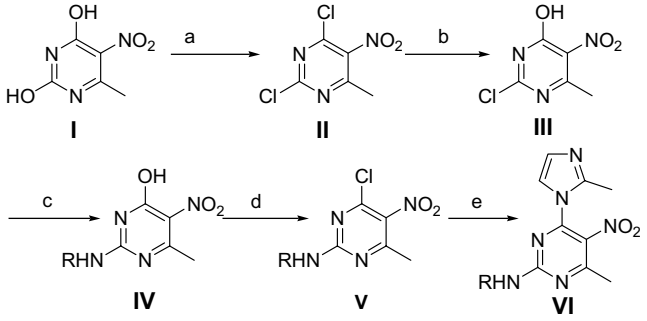
identification of UL70 as the viral target. Resistant virus cultivated in the presence of **1** carried a single point mutation of $P_{571} - S_{571}$. It was believed that the thiol group of cysteine 570 of the UL70 primase acts as a nucleophile that attacks compound **1** with displacement of the imidazole moiety. This results in the formation of a covalent link between the target enzyme and the drug, rendering the primase inactive (Fig. 1). Not surprisingly, early attempts to find a bioisosteric replacement of the imidazole, such as a C-linked 5-oxazole, 5-oxadiazole or 4-isoxazole, met with failure (Fig. 2). Removal of the imidazole or methyl imidazole resulted in a tremendous reduction in antiviral activity. Previous SAR

Table 1. Inhibition of hCMV replication (Dot blot assay[9]) by 2-substituted benzylamine analogs

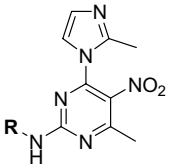


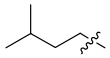
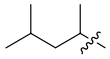
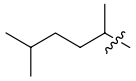
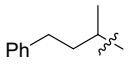
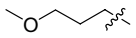
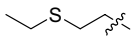
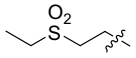
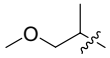
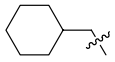
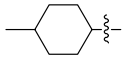
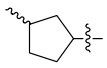
Compound	X	IC_{50} (μM)
6	2-CH_3	0.2
7	3-CH_3	0.5
8	4-CH_3	1.6
9	4-Cl	0.3
10	4-F	0.15
11	4-CF_3	0.2
12	3-F	0.25
13	3-OCF_3	0.3
14	3,5-di-CF_3	15
15	Ph=3-Py	0.8
16	2-OCF_3	0.2
17	3-OCF_3	0.3
18	3-OCH_3	0.3
19	3,4-di-F	0.4
20	2-Cl, 4-F	0.25
21	2-F, 4-CF_3	0.2
22	2,5-di-F	0.15
23	3,4-di-Cl	0.2
24	2,3-di-Cl	0.2
25	Ph=2-Py	0.5



Scheme 1. Reaction conditions for analog synthesis: (a) Et_4NCl, *N*, *N*-dimethylaniline, $POCl_3$, CH_3CN, 87%; (b) NaOAc, AcOH, EtOH, H_2O, 79%; (c) RNH_2, chloroform, reflux, 35–80%; (d) $POCl_3$, 100 °C, 70–85%; (e) 2-methyl imidazole, CH_3CN, 80 °C, 24 h, 95%.

Table 2. Inhibition of hCMV replication (Dot blot assay[9]) by 2-alkylamine analogs



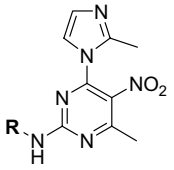
Compound	R	IC_{50} (μM)
26	H	2.0
27		1.0
28		0.5
29		0.3
30		0.13
31		2.3
32		0.35
33		2.3
34		0.2
35		1.3
36		0.9
37		0.6
38		0.2
39		0.12
40		0.2
41		0.15
42		0.15
43		0.08
44		0.1
45		0.5

studies also indicated that replacement of the nitro group with other electron-withdrawing groups also significantly diminished the antiviral activity. Analogs with 2-methyl imidazole had similar antiviral activity as analogs containing unsubstituted imidazole.[8]

Subsequent SAR studies were focused on close analogs of **1** by investigating the effect of substitutions on the phenyl ring while maintaining the methyl imidazole moiety intact. SAR results are summarized in Table 1. The antiviral activity (IC_{50}) was measured by using a Dot blot CMV DNA replication assay.[9] In general, the SAR trend is rather flat; there are no large increases in activity due to change of substitution patterns in this series. Most active inhibitors ($IC_{50} \leqslant 200$ nM) contain a small electron-withdrawing group on the phenyl ring. There is no additive SAR effect from multiple substitutions. Replacement of the electronically deficient phenyl ring in **10** by a pyridine group (compound **10** vs **15** and **25**) resulted in a slight reduction of antiviral activity. When compound **10** was dosed in rats, it showed relatively high clearance and poor oral bioavailability. Additionally, the majority of the compounds in Table 1 had poor aqueous solubility that prevented them from further in vivo evaluation.

We speculated that a sterically bulky alkylamino group could mimic the hydrophobic interactions of the benzyl group in the benzylamino series. Hindered secondary amino groups were expected also to be less metabolically labile than the benzylamino group toward oxidative dealkylation. The polarity of the alkylamino group

Table 3. Inhibition of hCMV replication (Dot blot assay[9]) by 2-cycloalkylamine analogs



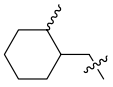
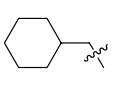
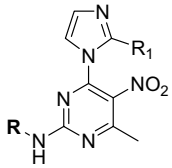
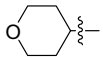
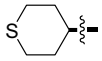
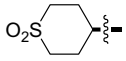
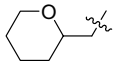
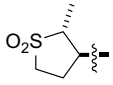
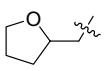
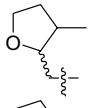
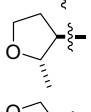
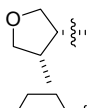
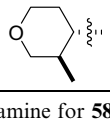
Compound	R	IC_{50} (μM)
46		0.1
47		0.7
48		0.09
39		0.12
49		0.3
50		1.5
51		0.1
52		0.15

Table 4. Inhibition of hCMV replication (Dot blot assay[9]) by 2-heterocyclo-amine analogs



Compound	R	IC_{50} (μM)
53		3
54		0.4
55		>5
56		4
57		0.4
58[a]		0.5
59[c]		0.15
60[c]		0.15
61		0.7
62		0.25
63		2
64		1
65		0.3
66[b]		0.6
67[c]		0.2
68		0.13

[a] For the synthesis of alkyl amine for **58** see Ref. 12.
[b] Synthesis of alkyl amine for **66** see Ref. 13.
[c] Synthesis of alkyl amines for **59**, **60**, and **67**, see Ref. 14.

Table 5. Antiviral activity, cellular toxicity, and bone marrow cellular toxicity comparison of **60**, GCV (ganciclovir), and CDV (cidofovir)

Compound	CMV replication[9] IC_{50} (μM)	CMV yield[17] $EC_{99.9}$ (μM)	HFF Tox.[15] CC_{50} (μM)	Jurkat Tox.[15] CC_{50} (μM)	CFU-GM Tox.[16] CC_{50} (μM)
60	0.13	0.4	100	50	50
GCV	1	5	>100	>100	30
CDV	0.35	0.9	100	100	6

could then be subsequently adjusted to increase aqueous solubility.

The general procedure for the preparation of this series of compounds is depicted in Scheme 1. Dichlorination of dihydroxypyrimidine **I** with phosphorooxytrichloride in refluxing acetonitrile produced 2,4-dichloropyrimidine **II** in 87% yield.[10] The 4-chloro atom in **II** was selectively removed under careful basic hydrolysis with NaOAc and HOAc to give **III**.[11] Amino substitution of 2-chloropyrimidine **III** in refluxing chloroform furnished compounds of general formula **IV** in 35–80% yields. The 4-chloro group of **V** was then reintroduced by reacting **IV** with phosphorooxytrichloride. The imidazole group was introduced by reaction with 2-methyl imidazole in refluxing acetonitrile to yield the final compounds **VI** (Scheme 1). All final compounds showed satisfactory ^{1}H NMR and LC mass spectral analysis. All the compounds are racemic except as noted.

Analogs with the proper alkylamine substituents display potent anti-CMV activity (Table 2). The antiviral activity increases as the size of the alkyl side chain increases (from **26** to **30**). However, if the substitution is too large like **31** or **32**, there is not further increase of antiviral activity. Polar groups, such as sulfone and ethers, are not very well-tolerated in the side chain (**33**, **35**, and **36**). The cyclohexyl analog (**38**) has good antiviral activity compared with the cyclopentyl analog (**37**). Additional substitution around the cyclohexyl ring, in particular, 2′-methyl substitution, provides the best antiviral activity (**43**). Only small alkyl-substituents on the 2′-position of the cyclohexyl ring are tolerated in this series.

Inhibitors with a methyl substitution at the 3′ or 4′ position of the cyclohexyl group are not as potent as the 2′ substituted analog. 2′-Methylcyclohexyl appears to be optimal in this series. Substituents larger than ethyl group (data not shown) compromise antiviral activity. For substituted cyclohexyl side chains, trans-substitution leads to better antiviral activity than cis-substitution. For example, *trans* compound **46** is sevenfold more potent than its *cis* isomer **47**. Most compounds containing cycloamino substituents at the 2-position of the pyrimidine ring still displayed poor (<5%) oral bioavailability in rats. In contrast to the in vitro glutathione reactivity of these compounds, glutathione replacement of the methyl imidazole or imidazole does not appear to be a major metabolic pathway in vivo. For example, compound **26** showed bioavailability of 59% after oral dosing of 2 mg/kg to rats. The poor oral bioavailability of the more potent analogs is likely due to their limited aqueous solubility, hence the poor absorption, and high rate of oxidative elimination in vivo. When compound **46** (Table 3) was incubated with rat liver S9 microsomes, the hydroxylation of the cyclohexyl group also appeared to be a major route of metabolism. Hence, it was necessary to design newer analogs with improved aqueous solubility and oxidative stability in order to obtain good oral CMV inhibitors.

To address this issue, we explored heterocyclic alkyl analogs (Table 4). Simple tetrahydropyran replacement of the cyclohexyl group resulted in a large decrease in antiviral activity (**53** vs **38**), while the more polar thiacyclohexane or thiacyclopentyl groups further decreased the antiviral activity (**55** and **63**). This observation is consistent with the earlier SAR trend that polar alkyl groups containing sulfone and ethers are not very well tolerated in the side chain. Fortunately, analogs with 2′-methyl substitution of the tetrahydropyran or the tetrahydrofuran group achieved similar potency as the 2′-methyl cyclohexyl analogs. Tetrahydrofuran or tetrahydropyran analogs had a good balance of potent antiviral activity, good aqueous solubility, and in vitro metabolic stability. Compounds **60** and **68** showed good pharmacokinetic profiles in rats with clearance values of 1.4 and 2.2 L/h/kg, and oral bioavailabilities of 21% and 57%, respectively. Additionally, compound **60** showed a much larger window between antiviral activity and cellular toxicity in the HFF, Jurkat,[15] and human bone marrow cells, when compared with ganciclovir and cidofovir (Table 5).[16]

Summary. A novel class of non-nucleoside CMV inhibitors has been identified. The compounds selectively and irreversibly bind to CMV UL70 primase, resulting in the blockade of CMV replication and infection. Detailed SAR studies on this series of CMV inhibitors yielded several potent and orally bioavailable drug candidates that have the potential to become a novel therapeutic approach to CMV infection.

Acknowledgment

We acknowledge the late Professor William H. Burns, Department of Medicine, Medical College of Wisconsin, for performing the bone marrow toxicity studies.

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7. The luciferase screen involved infection of human foreskin fibroblasts (HFF) with a recombinant virus (rCMVLUC) containing a luciferase reporter gene under the control of HCMV UL99 promoter, inserted in the CMV genome between US9 and US10. Luciferase activity was measured following incubation with various concentrations of the drugs.
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9. The Dot blot assay was used to determine the drug susceptibility against CMV. The foreskin fibroblast (HFF) cells were infected with cytomegalovirus (rCMVLUC). The infected HFF cells were incubated with various concentrations of the drug for 5 days. The rCMVLUCDNA was then harvested and immobilized on a membrane. The viral DNA was quantified using a radio-labeled probe containing CMV-specific sequences hybridized with the membrane. The IC_{50} values represent the drug concentration required to inhibit 50% of the amount of radioactivity captured by the membrane when the infected cells were treated only with drug vehicle.
10. (a) Clark, J.; Ramsden, T. *J. Chem. Soc., C* **1971**, 675; (b) Hanson, J.C. PCT Int. Appl. WO 91/01310. see also Hodgetts, K.J.; Yoon, T.; Huang, J.; Gulianello, M.; Kieltyka, A.; Primus, R.; Brodbeck, R.; De Lombaert, S.; Doller, D. *Bioorg. Med. Chem. Lett*. **2003**, 13(15), 2497.
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12. The starting amine was synthesized by azide replacement of the corresponding alcohol synthesized by the method described in reference (Amouroux, R.; Slassi, A.; Saluzzo, C. *Heterocycles*, **1993**, 36(9) 196), followed by hydrogenation.
13. The starting amine was prepared similarly to examples in Ref. 12 starting with the same alcohol followed by Mitsunobu reaction with *p*-chlorobenzoic acid and followed by hydrolysis, azide replacement, and reduction.
14. The starting amine was prepared by methyl Grignard reagent opening of epoxide catalyzed by CuI, azide replacement of the alcohol, and azide reduction. The enantiomerically pure amine was prepared by chiral resolution with D- or L-malic acid recrystallized in ether. The absolute stereo chemistry was determined by X-ray crystallography of the crystal of 4-iodo-*N*-((3*R*,4*S*)-4-methyl-tetrahydrofuran-3-yl)benzeneulfonamide and 4-iodo-*N*-((3*S*,4*R*)-4-methyl-tetrahydrofuran-3-yl)benzeneulfonamide.
15. The cytotoxicity assay in HFF or Jurkat cells was performed by Alamar blue assay. HFF or Jurkat cells were seeded into 96-well plates and then incubated with various concentrations of 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). The IC_{50} value represents the concentration of compound that inhibits 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 with 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 to 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. The viral yield from the rCMVLUC-infected HFF cells was determined by the immunofluorescence assay using human antiserum to HCMV after 5–7 days incubation in the presence of various concentrations of the drug.