Discovery of Potent, Orally Bioavailable Inhibitors of Human Cytomegalovirus

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S Supporting Information

ABSTRACT: A high-throughput screen based on a viral replication assay was used to identify inhibitors of the human cytomegalovirus. Using this approach, hit compound **1** was identified as a 4 μ M inhibitor of HCMV that was specific and selective over other herpes viruses. Time of addition studies indicated compound **1** exerted its antiviral effect early in the viral life cycle. Mechanism of action studies also revealed that this series inhibited infection of MRC-5 and ARPE19 cells by free virus and via direct cell-to-cell spread from infected to uninfected cells. Preliminary structure–activity relationships demonstrated that the potency of compound **1** could be improved to a low nanomolar level, but metabolic stability was a key optimization parameter for this series. A strategy focused on minimizing metabolic hydrolysis of the N1-amide led to an alternative scaffold in this series with improved metabolic stability and good pharmacokinetic parameters in rat.

KEYWORDS: HCMV, cell-to-cell spread, replication inhibitors, antiviral agents

Although infection with human cytomegalovirus (HCMV) does not lead to disease in healthy individuals, it can cause end-organ disease in the fetus, in the allograft recipient, in bone marrow transplant patients, and in AIDS patients.¹ Since HCMV infects approximately 60% of people in the developed world and over 99% in developing countries, the risk of morbidity and mortality due to HCMV disease is a significant problem for public health on a global scale.¹ Furthermore, a recent study has linked HCMV infection to increased mortality in the general population in the US. The current gold standard therapy for HCMV infection is either gancyclovir or its prodrug valgancyclovir.² However, when resistance or poor tolerability arise with these medications, second line options frequently suffer from lack of efficacy due to poor bioavailability, modest potency, and poor tolerability due to their toxicity at efficacious exposures.² Thus, a significant gap in treatment options exists, which has led to substantial efforts to develop new drugs to treat this opportunistic infection. There has been a sustained effort to discover an effective HCMV vaccine for the prevention of HCMV infection and although there have been positive outcomes in a number of clinical proof of concept studies, no marketed vaccine yet exists.³ Currently, the leading edge in the discovery of new small molecule therapeutic agents includes the ongoing development of maribavir, letermovir, and brincidofovir, inhibitors of UL97, terminase, and HCMV polymerase,

respectively.¹ These compounds have all demonstrated positive clinical proof of concept in phase II studies and may represent the next generation of therapy options for patients that have or are at risk of developing HCMV disease.

Due to the significant unmet medical need for patients at risk for HCMV-related end organ disease, a drug discovery effort aimed at discovery of potent and selective inhibitors of HCMV replication was initiated at Boehringer Ingelheim. In the search for new lead matter, an HTS was conducted using a viral replication assay that measured the cytopathic effect upon infection of MRC-5 cells with the AD169 laboratory strain of HCMV. A diversity-based subpool of the BI corporate compound collection totaling 97,733 compounds was screened and returned 1424 compounds for a hit rate of 1.5%. Following hit confirmation and determination of EC₅₀ values⁴ of the confirmed actives, compounds were tested for lack of potency against replication of human immunodeficiency virus, hepatitis C virus, and human rhinovirus in order to establish the specificity for HCMV of each hit cluster. Cytotoxicity was assessed in a variety of cell types and all compounds with dose responsive effects were removed from the hit set. Finally, the

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potency against the TR clinical isolate of HCMV was determined as a rule-in assay. Following this hit triage, a single cluster remained, exemplified by the most active member of the series, compound **1**.

Compound **1** was evaluated in a number of antiviral and in vitro ADME assays, and the resulting profile is outlined in Table 1. Compound **1** is characterized by micromolar potency

Table 1. Profile of Compound **1**

assay	result
EC ₅₀ (AD169) CPE ^a	4.0 μM
EC ₅₀ (AD169) qPCR ^b	2.5 μM
EC ₅₀ (TR strain) CPE	8.2 μM
CC ₅₀ (MRC-5)	>82 μM
t _{1/2} , HLM	12 min
P _{app} , Caco-2	11 × 10 ⁻⁶ cm/s
solubility (pH = 6.8)	1.9 μg/mL
IC ₅₀ CYP450 inhibition (3A4) (2D6)	>30 μM

^aHalf maximal effect concentration measured by cytopathic effect for infection of MRC-5 cells with AD169 train of HCMV. ^bHalf maximal effect concentration measured by RT-qPCR⁵ for infection of MRC-5 cells with AD169 train of HCMV.

in a range of viral replication assays⁵ with acceptable windows versus cytotoxicity. Compound **1** demonstrated a Caco-2 permeability of P_{app} = 11 in the apical to basolateral direction and did not show significant inhibition of a selected panel of CYP450 isoforms. Key optimization parameters for this compound include metabolic stability when incubated with human liver microsomes (HLM) and relatively poor aqueous solubility, which was measured at 1.9 μg/mL at pH = 6.8. Based on this overall profile, hit evaluation aimed at establishing the suitability of this chemotype for further optimization was initiated.^{6,7}

Since the molecular target of this series of replication inhibitors was unknown, development of early structure–activity relationships (SARs) relied on the viral replication assay used in the primary screen. In these early explorations, focused amide libraries at the N1 and N4 positions were prepared and two key findings from this effort are summarized in Table 2. First, it was found that the N1 position showed very steep potency SARs, and generally only a very select number of five-membered heterocycles were tolerated as replacements for the furan moiety without a complete loss in antiviral potency, with the 4-thiazolyl group generally providing the most active compounds (cf. compounds **2–5**). Second, the N4 position was shown to be very permissive to structural changes and we were quickly able to improve antiviral potency to the submicromolar range through introduction of more lipophilic substituents (cf. compounds **1** and **6–9** or compounds **2** and **10**). Another early observation was that the poor metabolic stability could be modulated by the nature of the N1 substituent, a finding that would become important later in the optimization effort. Based on these initial indications of tractable SAR, an optimization effort aimed at aligning favorable potency and drug-like properties was initiated.

During the course of hit evaluation, we noted the structural similarity of our inhibitors to a series of HCMV replication inhibitors exemplified by compound **11** (CFI02), previously disclosed by scientists at Wyeth (now Pfizer). In their accounts of the discovery⁸ and study⁹ of these compounds, the authors

Table 2. R1 and R4 SARs

	R1	R4	^a EC ₅₀ (μM)	^b CC ₅₀ (μM)	^c HLM t _{1/2} (min)
1			4.0	>82	12
2			0.64	>57	22
3			4.4	>57	98
4			3.0	>57	34
5			>57	>57	n.a.
6			0.34	>57	14
7			1.1	>57	14
8			8.0	>57	n.a.
9			38	>57	n.a.
10			0.22	>57	94
11			0.25	36	110

^aHalf maximal effect concentration measured by cytopathic effect for infection of MRC-5 cells with AD169 train of HCMV. ^bHalf maximal cytotoxic effect concentration on MRC-5 cells. ^cHalf-life.

noted that the compounds acted at a very early step in the viral life cycle and speculated that the molecular target might be glycoprotein B, a viral envelope protein required for efficient fusion of viral particles with host cells. Time of addition studies performed with compound **1** and **11** in a luciferase-based single cycle of infection assay were consistent with a similar mechanism of action between the two series of compound

(Figure S1). However, extensive effort to raise resistance to the BI series of compounds or identify the target through photo-cross-linking experiments was unsuccessful, and so it remains unknown if the target of these compounds is glycoprotein B, another viral protein or a host cell protein.

The compounds featured in Table 2 all have an embedded 1,4-diaminobenzene core capped on each nitrogen atom with an acyl group. If either of these amides were subject to hydrolysis *in vivo*, the resulting electron rich aniline could potentially be subject to bioactivation, which in turn could pose a risk of genotoxicity.¹⁰ In view of this consideration, HLM-metabolite identification experiments were conducted to establish if either amide bond was generally predisposed to metabolic hydrolysis. The results clearly indicated that the N1 amide bond was hydrolyzed to give the corresponding aniline. A range of amide replacements was prepared and tested, and in all cases, a complete loss in antiviral potency was observed, indicating that this structural feature was crucial for potency. A wide variety of aliphatic replacements for the central aromatic core were also screened, but in all cases explored, a complete loss of antiviral potency was observed.

Faced with the reality that the N-aryl amide core of this series was a required pharmacophoric element of these inhibitors, the specific structural requirements needed for metabolic hydrolysis of the N1 amide were established empirically using the HLM assay. In short, the metabolic stability for a wide range of compounds was compared in the presence and absence of the cytochrome P450 cofactor NADPH (a full description can be found in the Supporting Information). Compounds were grouped based on the dependence of metabolic stability on the presence of NADPH in the assay conditions. Structural features common to the compounds that depended on NADPH for metabolic clearance were then prioritized for future optimization. From this analysis, two general trends emerged. First, the nature of the five-membered heteroaromatic substituent had a direct impact on metabolic hydrolysis (Figure S3A). For example, the furan and oxazole substituent, found in compounds 1 and 3, respectively, were completely resistant to hydrolysis, while both the thiazole and thiadiazole found in compounds 2 or 4, respectively, were particularly prone to hydrolysis. Unfortunately, aligning potency with ADME properties favorably for the furan or oxazole containing compounds was not possible, and so a path forward based on modification of this ring was not feasible. Second, the nature of the embedded aromatic amine played a significant role in modulating the susceptibility to metabolic hydrolysis (Figure S3B). A summary of the core replacements that were identified that maintain antiviral potency and the corresponding risk of hydrolysis are found in Table 3. In most cases, these core modifications gave submicromolar IC₅₀ values in viral replication assays within 5-fold of each other and so represented options for future optimization. The risk of genotoxicity for each core modification was also evaluated through a consensus approach involving (a) predicting the AMES mutagenicity of the corresponding aromatic amine using DEREK¹¹ and (b) experimental data (in house or literature) on related aromatic amines.

Aniline based cores represented by compounds 10 and 12–14 all showed a clear propensity for metabolic hydrolysis and substantial risk of genotoxicity and so were deprioritized (Figure S2). In contrast, the cores represented by compounds 15–18 did not show susceptibility to hydrolysis, but carried some risk of genotoxicity. Only the pyridone core represented by

Table 3. Core Modifications and Assessment of Risk for Amide Hydrolysis and Genotoxicity

	X	^a EC ₅₀ (μM)	^b CC ₅₀ (μM)	Hydrolysis Risk ^c	Genotoxicity Risk ^d
10		0.21	>57	+	+++
12		0.13	36	+	+++
13		0.13	7.8	+	+++
14		0.43	>57	+	+
15		0.54	>57	-	+
16		0.44	>57	-	+
17		2.3	>57	-	+++
18		0.25	>57	-	+
19		0.44	28	-	-

^aHalf maximal effect concentration measured by cytopathic effect for infection of MRC-5 cells with AD169 train of HCMV. ^bHalf maximal cytotoxic effect concentration on MRC-5 cells. ^cSee scoring in Figures S2 and S3. ^dSee Table S1.

compound 19 was completely free of a risk of hydrolysis and genotoxicity and so became the focus of optimization.

The acetamide linker found in the structure of compound 19 was modified in a number of different ways. Linkers that incorporate a heteroaromatic group demonstrated clear SAR and were the most attractive option identified for introduction of structural diversity. For example, while compound 20 was inactive at the highest concentration tested, methylation at the benzylic position (compound 21) restored antiviral potency. The regioisomeric imidazole 22 displayed improved antiviral potency but also showed a substantial increase in its ability to

Table 4. SARs at the R⁶ Position

	R	^a EC ₅₀ (μM)	Solubility pH = 6.8 (μg/mL)	^b HLM t _{1/2} (min)	CYP450 Inhibition IC ₅₀ (μM)		
					3A4	2C8	2C9
20		>5.8	<0.16	>120	1.9	n.a.	2.1
21		2.4	<0.10	n.a.	n.a.	1.7	2.1
22		0.15	<0.10	>120	>30	n.a.	0.031
23		0.47	n.a.	>120	>30	4.3	7.3
24		0.54	76	>120	>30	4.7	3.0
25		3.6	63	n.a.	>30	21	13
26		1.9	16	72	>30	18	14
27		0.028	<0.10	16	>30	n.a.	0.15
28		0.11	<0.10	>120	>30	0.18	1.4

^aHalf maximal effect concentration measured by RT-qPCR of MRC-5 cells with AD169 train of HCMV; see [Supporting Information](#). ^bHalf-life.

inhibit the 2C9 CYP450 isoform. This could be partially offset by addition of a hydroxyl group at the benzylic position to give compounds **23** and **24**, which gave inhibitors with similar antiviral potency and 235- and 97-fold increases, respectively, in the IC₅₀ value for CYP450 2C9. Increasing polarity of these inhibitors did not generally lead to decreases in CYP450 inhibition. However, a dependence of CYP450 2C9 inhibition on the substitution pattern found on the phenyl ring was observed, as exemplified by a comparison of compound **24** to compounds **25** and **26**, but in general, losses in potency for CYP450 2C9 inhibition paralleled losses in antiviral potency. Antiviral potency could be further improved by extension of the alkyl substituent of compound **24** to give, for example, compound **27** or by alkylation of the hydroxyl group of compound **23** to give, for example, compound **28**. However, these types of modifications were accompanied by increases in

the ability of the compounds to inhibit either CYP450 2C9 or 2C8. In addition to benefiting the CYP450 inhibition profile, introduction of the hydroxyl group found at the benzyl position to give compounds **24–26**, and related analogues also resulted in improved solubility at pH = 6.8 and good half-lives when incubated with human liver microsomes (Table 4).

Infection of host cells by viral particles can depend on different glycoprotein complexes to different extents, an important concern for inhibitors that may exert their mechanism of action at the fusion step of viral entry.¹² Therefore, compound **24** was screened against a panel of clinical isolates (Table 5) where it was generally found that this compound inhibit the infection of MRC-5 cells (a human lung fibroblast lineage) by the TR, TB40 and VR1814 strains with potency comparable to that of the AD169 lab strain. This compound also inhibited infection of ARPE-19 cells (a human

Table 5. Expanded Profile of Compound 24

EC ₅₀ ^a	AD169/MRC-5, μ M	0.54
	TB40/MRC-5, μ M	0.25
	TR/MRC-5, μ M	0.47
	VR1814/MRC-5, μ M	0.91
	VR1814/ARPE-19, μ M	1.7
	VR1814/ARPE-19 (cell-to-cell spread) μ M	0.80
solubility	(pH = 6.8), μ g/mL	76
metabolic	HLM, $t_{1/2}$	>120
stability	HHEP %QH	17
	RHEP %QH	9.5
IC ₅₀ Cyp450	(3A4/2C8/2C9/2D6), μ M	>30/4.7/3.0/17
rat PK	Cl, %QH	24
	$t_{1/2}$, h	1.9
	V _{ss} , L	2.5
	%F	72

^aHalf maximal effect concentration measured by RT-qPCR.

retinal epithelial lineage) by the VR1814 strain at a similar level of potency. In the qPCR-based antiviral assays, compounds are added at the same time as the virus, and due to viral kinetics, inhibition of only one cycle of viral replication is evaluated. However, when multiple rounds of infection are possible, such as in the clinical setting, HCMV is believed to spread not only via newly formed viral particles but also through direct cell-to-cell spread involving adjacent cells.¹³ In order to confirm that our series of replication inhibitors could prevent this process from occurring, we developed an assay designed to restrict spread of VR1814 from an infected ARPE-19 cell to an adjacent, uninfected ARPE-19 cell. In short, cells are infected and viral replication was allowed to proceed for 24 h. The media and accompanying viral particles were removed, and Cytogam, a CMV neutralizing antibody that does not prevent cell-to-cell spread,¹⁴ is added along with new assay media. After a further 48 h, which allows for one full cycle of replication to occur, compounds were added. After a final 72 h, nuclei of infected cells can be differentiated from uninfected cells by staining with a fluorescent antibody that recognizes the HCMV immediate early protein (IE1). A representative example of the dose responsive effect of one of our inhibitors of viral replication (IC₅₀ = 134 nM, VR1814/ARPE-19, qPCR) on cell-to-cell spread in this assay is given in Figure 1. At low concentration, clusters of adjacent infected cells are visible, which would correspond to a plaque in a classical plaque reduction assay. In contrast, at high compound concentration, single infected cells, which are the result of the single cycle of replication that transpires before compounds are added, are visible and are relatively distant from other infected cells. Using these images, dose response curves were generated (see Supporting Information for full details) and gave an IC₅₀ value of 128 nM for this compound. In general, IC₅₀ values determined with this assay show excellent correlation with our routine qPCR assays (VR1814/APRE-19 and AD169/MRC-5), and taken together, the results indicate that our series of replication inhibitors efficiently block the direct cell-to-cell spread of HCMV. The *in vitro* ADME profile of compound 24 is also collected in Table 5. Compound 24 also shows improved solubility and is stable when incubated with human liver microsomes and human or rat hepatocytes. Compounds 24 shows a CYP450 inhibition profile characterized by no significant inhibition of the 3A4 isoform and modest inhibition

Figure 1. Dose responsive effect of HCMV inhibitor on direct cell-to-cell spread of VR1814 on ARPE-19 cells.

of the 2D6, 2C8, and 2C9 isoforms. Evaluation of compound 24 in rat pharmacokinetic experiments revealed favorable volume of distribution, oral bioavailability, and low clearance, which was in line with the stability observed in the rat hepatocyte assay.

In conclusion, a phenotypic screening approach was used to discover a single series of HCMV replication inhibitors represented by hit compound 1. Time of addition studies indicate that this series may share the same mechanism of action as CFI02, a compound reported to be a HCMV fusion inhibitor. A novel cell-to-cell spread assay was developed and used to show that our series of compounds inhibit cell-to-cell spread of HCMV as efficiently as they inhibit infection of host cells by free viral particles. Preliminary exploration around compound 1 indicated steep SAR at the N1 position, which was also the site of NADPH independent metabolism when incubated with human liver microsomes. Flexibility toward modification of the aromatic core and linker regions of the molecules allowed for the prioritization of scaffolds with low propensity for metabolic hydrolysis of the N1 amide, which in turn limits the potential for liberation of a genotoxic metabolite. Finally, continued exploration of the linker region of the molecule allowed for the identification of compound 24, which is characterized by improved potency, solubility and metabolic stability, and favorable rat pharmacokinetic profiles, providing a high quality starting point for a lead optimization campaign targeting the development of a new therapeutic option for patients with or at risk of developing HCMV disease.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00064.

Characterization data for key compounds, scoring of substructures for metabolic hydrolysis, time of addition experiments, and protocol for cell-to-cell spread assay (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HCMV, human cytomegalovirus; HTS, high-throughput screen

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