Peptidomimetic Inhibitors of Herpes Simplex Virus Ribonucleotide Reductase with Improved *in Vivo* Antiviral Activity

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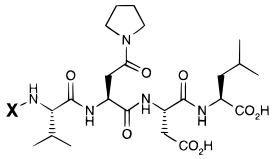
We have been investigating the potential of a new class of antiviral compounds. These peptidomimetic derivatives prevent association of the two subunits of herpes simplex virus (HSV) ribonucleotide reductase (RR), an enzyme necessary for efficient replication of viral DNA. The compounds disclosed in this paper build on our previously published work. Structure−activity studies reveal beneficial modifications that result in improved antiviral potency in cell culture in a murine ocular model of HSV-induced keratitis. These modifications include a stereochemically defined (2,6-dimethylcyclohexyl)amino N-terminus, two ketomethylene amide bond isosteres, and a (1-ethylneopentyl)amino C-terminus. These three modifications led to the preparation of BILD 1351, our most potent antiherpetic agent containing a ureido N-terminus. Incorporation of the C-terminal modification into our inhibitor series based on a (phenylpropionyl)valine N-terminus provided BILD 1357, a significantly more potent antiviral compound than our previously published best compound, BILD 1263.

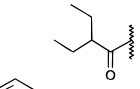
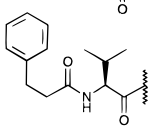
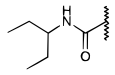
Introduction

We have been pursuing a new class of antiviral compounds for their potential utility as inhibitors of herpes simplex virus (HSV) replication. These compounds prevent association of the two subunits of HSV ribonucleotide reductase (RR), an enzyme that catalyzes the conversion of ribonucleotide diphosphates into the corresponding deoxyribonucleotides, thereby preparing the DNA building blocks required for HSV genomic replication.[1] Our subunit association inhibitors are based on the C-terminal amino acid sequence of the HSV RR small subunit (R2). This sequence binds to the RR large subunit (R1) and consequently permits subunit association and subsequent catalytic activity.[2] Our inhibitors, which mimic this C-terminal sequence, compete with R2 for binding to R1. This mechanism of inhibiting HSV RR is attractive because compounds based on the HSV R2 C-terminal sequence do not inhibit host cell RR.[3] Despite the fact that herpes and human RRs function by the same mechanism and have similar structural features, the critical R2 C-terminal regions of these enzymes have little sequence homology. Consequently mimics of either enzyme's R2 C-terminus do not cross-inhibit the other ribonucleotide reductase.[4]

Given that our inhibitors mimic an amino acid sequence, these compounds have thus far maintained a degree of peptidic character. The suitability of peptide- and peptidomimetic-based compounds as effective drugs remains an important question.[5] As an important first step, we recently demonstrated that inhibitors of HSV RR subunit association could not only prevent HSV replication in cell culture but also reduce the severity of HSV-induced keratitis in a murine ocular model.[6] This work provided the first illustration that peptidomimetic compounds can prevent enzyme subunit association *in vivo.* In this report, we detail our continuing efforts to identify new inhibitors of HSV RR that are more potent antiherpetics *in vitro* and *in vivo.*

Table 1



Compound *	X	Binding Assay IC_{50} (nM)
1		860 ± 240
2		24 ± 5
3		15 ± 1

* Enzyme assay IC_{50} of Val-Val-Asn-Asp-Leu, 760,000 nM.[7]

Our structure−activity studies are based on the five C-terminal amino acids of HSV R2 (Val-Val-Asn-Asp-Leu), since this sequence contains the most important functionalities for binding to HSV R1.[7] Table 1 depicts three classes of RR inhibitors that we have previously published. The distinction between these three classes lies at the inhibitor N-terminus. Our first structure−activity disclosure, compound **1** being a representative example, highlighted the role and relative importance of the various amino acid side-chain functionalities for potency in an HSV RR enzyme assay.[8] However, none of the compounds in this paper proved efficacious against HSV in cell culture. Progress toward efficacy in HSV cell culture was obtained with the inhibitor series represented by compound **2**.[9] A binding interaction between the phenyl ring in **2** and R1 accounted for the 30-fold increase in binding assay potency over

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compound **1**. Incorporation of various beneficial modifications into compound **2** eventually led to the discovery of BILD 1263 (Table 7), a potent inhibitor of HSV RR subunit association (IC_{50} = 0.2 nM) that also inhibited the replication of HSV types 1 and 2 in cell culture (EC_{50} = 3 and 4 μM). It was this molecule that became the first RR subunit association inhibitor to reduce the severity of HSV induced keratitis in a murine ocular model.[6]

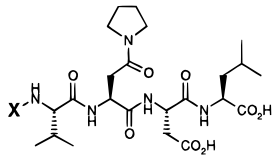
More recently, we revealed that a minor modification to our first published inhibitor series, represented by compound **1**, could increase binding potency to the same level as the inhibitor series represented by compound **2**. Insertion of an NH group near the N-terminus of compound **1** improved binding potency over 50-fold (cf. compounds **1** and **3**).[10] Evidence led us to conclude that this increase in binding potency was due to a hydrogen-bonding interaction between R1 and the NH group. The inhibitor series represented by **3** also provided a point of reference for a series of conformationally restricted inhibitors designed to probe the bioactive conformation of these peptide-based compounds. However, this publication did not describe the antiviral properties of these new ureido-based inhibitors. Herein, we reveal modifications to the inhibitor series represented by compound **3** that have proven beneficial for *in vitro* and *in vivo* antiviral potency. Although the molecules about to be discussed do not represent an exhaustive list of modifications investigated, they do include the most important ones identified so far. This paper provides a summary of the structure−activity studies and the thought process that led to the discovery of these inhibitors.

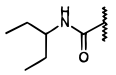
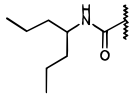
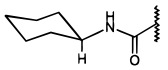
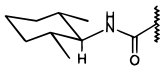
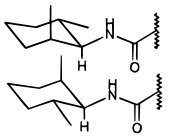
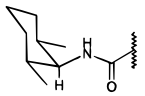
Before discussing the new results, it is important to point out a protocol that has often proven necessary to help assess the effect of new inhibitor modifications. Binding assay potency has proven to be an important criterion for good potency in HSV cell culture.[9] The radioligand binding assay used to determine the potency of our inhibitors against HSV RR is reliable for compounds with IC_{50}s of around 1 nM or higher.[9,11] However, virtually all compounds that have good cell culture potency are believed to have true IC_{50}s substantially lower that 1 nM. This makes assessment of the effect of a new modification on binding potency difficult if we use our most potent molecules as a point of reference for further structure−activity studies. Consequently, we frequently investigate new structural modifications in a less potent series first. Modifications that improve binding potency or alter physicochemical properties without significantly lowering binding potency are then incorporated into our most potent inhibitors for evaluation in HSV cell culture. This protocol is used in this paper to illustrate the effects of the new modifications about to be discussed.

Results and Discussion

Modification of the N-Terminal Lipophilic Group. The presence of the 1-ethylpropyl N-terminus in compound **3** is very important for good binding potency. Replacement of this group with an isopropyl moiety lowers potency approximately 40 times (compound **5**, Table 2), and further truncation to a methyl group results in a 2800-fold loss of binding potency.[10] Conversely, replacement of the 1-ethylpropyl group in **3** with a larger lipophilic group improved binding potency 2-fold (cf. compounds **3** and **4**). The importance of the N-terminal lipophilic group in inhibitors **3** and **4** for good binding potency, combined with the assumed inherent flexibility of these alkyl chains, raised the potential that locking of these chains in a favorable orientation for binding to R1 might improve inhibitor potency. However, it was not immediately obvious how to achieve this.

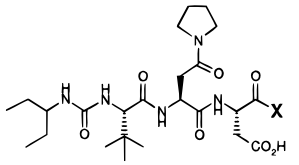
Table 2

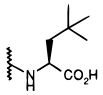
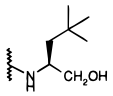


Compound	X	Binding Assay IC_{50} (nM)
3		15 ± 1
4		7 ± 1
5		440 ± 40
6		500 ± 50
7		55 ± 7
8		20 ± 2
9		2 ± 0.2

Through screening of various readily available alkylamines at the inhibitor N-terminus, we noticed the similar potencies of inhibitors containing either a cyclohexylamino or isopropylamino group (cf. compounds **5** and **6**, Table 2). On the basis of the assumption that the N-termini of **5** and **6** bound to R1 similarly, the equivalent potencies of these inhibitors implied that much of the cyclohexyl ring in **6** did not interact with R1. This suggested the use of the cyclohexyl ring as a semirigid scaffold for orienting alkyl groups in specific directions. Adding two methyl groups to the cyclohexane ring as shown in compound **7** improved binding potency 10-fold. Moreover, changing the stereochemistry of one of the methyl groups, as shown for the compound **8** mixture, further improved the binding potency to the level of compound **3**. Since these results showed that both equatorial or axial methyl groups on the cyclohexane ring could improve binding potency, the possible benefit of a lipophilic group that could occupy both equatorial and both axial positions became an obvious extrapolation. One N-terminal group that achieved this general arrangement of alkyl functionality

Table 3



Compound	X	Binding Assay IC_{50} (nM)
10		0.6 ± 0.2
11		6 ± 1
12		100 ± 10
13		350 ± 50
14		40 ± 6
15		1900 ± 200
16 R=Me **17** R=Et		27 ± 5 18 ± 2
18 R=Me **19** R=Et		2700 ± 100 2000 ± 400

was the (dimethylcyclohexyl)amino group shown in compound **9**. The two methyl groups on the cyclohexane ring forced the cyclohexane ring to adopt the conformation shown in Table 2. NMR coupling constants provided evidence for the predominance of this conformation in solution (coupling constant of 10, 3, and 3 Hz for the hydrogen adjacent to the N-terminal nitrogen). Importantly, compound **9** turned out to be at least 10-fold more potent than isomers **7** and **8** and more potent than either compounds **3** or **4**.

The lipophilic N-terminal group in compound **9** constitutes the most potent substitution identified at this position so far. More significantly, this particular (dimethylcyclohexyl)amino group also provided inhibitors with improved *in vivo* potency. This point will be further elaborated later.

Modification of the Inhibitor C-Terminus. Our previous structure−activity studies show that replacement of the C-terminal carboxylic acid with a hydroxymethyl group lowers binding potency 7−10-fold.[8,9] Compounds **10** and **11** in Table 3 further illustrate this observation. However, inhibitors containing a hydroxymethyl C-terminus are up to 10 times more potent in cell culture than the corresponding carboxylic acid analogue.[9] These results demonstrate the important relationship between inhibitor physicochemical properties and cell culture potency. Polar functionality such as carboxylic acids may impede the ability of inhibitors to reach efficacious concentrations inside cells. With this in mind, we sought to further reduce hydrophilicity at the inhibitor C-terminus by removing the hydroxymethyl group. The binding potency of the resultant inhibitor, compound **12**, drops a further 15-fold, and unfortunately, molecules containing the (3,3-dimethylbutyl)amino C-terminus do not look promising in cell culture.

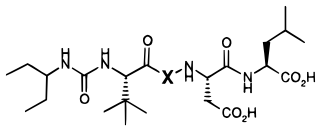
We previously showed that replacement of a leucine residue at the inhibitor C-terminus with an alanine lowered inhibitory potency over 2000-fold.[8] This result demonstrated the importance of the C-terminal lipophilic group and suggested that this region of the inhibitor might be participating in a relatively precise binding interaction with R1. Therefore, we were somewhat surprised to discover that a neopentylamine C-terminus produced an inhibitor more potent than the corresponding (3,3-dimethylbutyl)amino analogue (cf. compounds **12** and **14**). Also surprising was the necessity of all three methyl groups of the neopentylamine for good binding potency. Unlike compound **12** where removal of one methyl group lowered binding potency only 3.5 times,[12] removal of one methyl group from compound **14** lowered binding potency almost 50-fold (cf. compounds **12** with **13** and **14** with **15**). These results indicated the potential existence of multiple binding modes for the C-terminal lipophilic groups of various inhibitors. More significantly, inhibitors containing the neopentylamine C-terminus consistently had similar cell culture potencies to those containing the synthetically more complex γ-methylleucinol (see compounds **27** and **28**, Table 6 for one example).

Despite the appealing simplicity of the neopentylamine C-terminus, we also explored the potential of more elaborate neopentylamine derivatives. One of the most beneficial modifications resulted from the introduction of a stereochemically defined methyl or ethyl group α to the C-terminal nitrogen. Substituted neopentylamines possessing an (*R*)-alkyl group, compounds **16** and **17**, were at least two times more potent than the parent compound **14**, while the corresponding *S*-analogues **18** and **19** were approximately 50-fold less potent. Although the binding potency increases associated with the addition of an (*R*)-alkyl group were not large, the resultant improved cell culture potencies maintained our interest in these neopentylamine derivatives (see compounds **27** and **29**, Table 6 for one example).

The Bis-Ketomethylene Isostere. The advent and incorporation of amide bond isosteres into peptide and peptidomimetic compounds is an area of active investigation. Removal of an amide bond can potentially increase metabolic stability and change physicochemical properties which can favorably affect cellular uptake. With the knowledge that amide bonds provide sites for hydrogen bonding to water, we hoped that removal of heteroatoms associated with an amide bond might decrease the desolvation energy required for passive diffusion through the cell's lipid bilayer and thus improve cell culture potency. Before testing this hypothesis, we first investigated amide linkages that could be modified with minimal effect on binding assay potency.

One amide bond that could be successfully modified is located between the pyrrolidine-modified asparagine and *tert*-leucine residues (see Table 4). We previously

Table 4



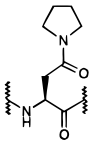
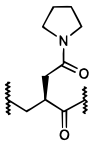
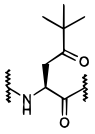
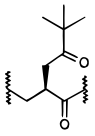
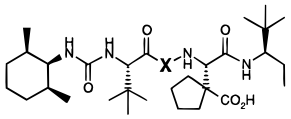
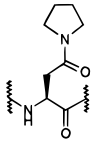
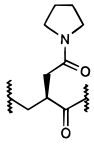
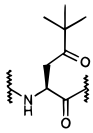
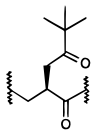
Compound	X	Binding Assay IC_{50} (nM)
20		3 ± 0.5
21		7 ± 1
22		3 ± 1
23		5 ± 1

Table 5



Compound *	X	Cell Culture Assay EC_{50}(μM) HSV-1	HSV-2
24		28 ± 13	40 ± 6
25		50 ± 13	80 ± 25
26		13 ± 6	13 ± 4
BILD 1351		6 ± 2	7 ± 4

* All compounds have binding assay IC_{50}s below 1 nM

demonstrated that the pyrrolidine-modified asparagine backbone NH was likely not involved in a binding interaction with R1 since it could be alkylated with no adverse effect on inhibitor binding potency.[10] This led us to replace this NH with a CH_2 (a ketomethylene isostere, cf. compounds **20** and **21**, Table 4). We were pleased to discover that this modification had little effect on inhibitor binding potency.

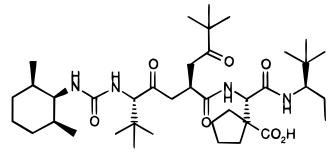
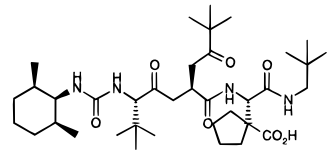
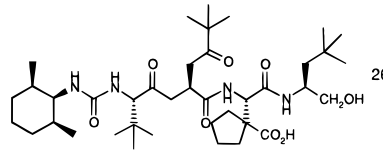
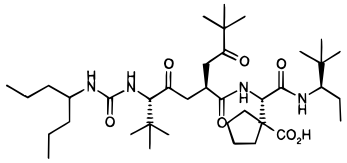
The side-chain amide of the pyrrolidine-modified asparagine residue could be modified in a similar manner. We previously demonstrated that dialkylation of the asparagine side-chain nitrogen (pyrrolidine found to be the best example) improved inhibitor binding potency 100-fold over the unsubstituted nitrogen.[8,9] Attempts to alter the shape of this group, with the optimistic intention of increasing binding potency, led to the preparation of the corresponding *tert*-butyl derivative (compounds **22** and **23**). Although the compounds shown in Table 4 showed that replacement of the pyrrolidine moiety with a *tert*-butyl group had virtually no affect on binding potency, we had removed one amide bond, and more significantly, inhibitors containing a *tert*-butyl group proved less hydrophilic than the corresponding inhibitor containing a pyrrolidine.[13]

Inhibitor Cell Culture Potency. Incorporation of the new N- and C-terminal modifications just discussed into inhibitors containing cyclopentylaspartic acid, a modification previously shown to improve both binding and cell culture potencies,[9] led to the preparation of compound **24** (Table 5). Unlike the inhibitors shown in Tables 1−4, compound **24** inhibited the replication of HSV-1 and -2 in cell culture below 1000 μM (EC_{50} 28 and 40 μM). This molecule demonstrated that inhibitors containing a ureido N-terminus could inhibit HSV replication in cell culture. Previously, only inhibitors based on the larger (phenylpropionyl)valine N-terminus, e.g. BILD 1263, showed efficacy in cell culture.

Further attempts to improve the cell culture potency of the ureido-based inhibitors led to the disappointing discovery that replacement of the amide bond between the *tert*-leucine and the pyrrolidine-modified asparagine residues with a ketomethylene isostere did not, as predicted, improve potency (cf. compounds **24** and **25**, Table 5). Nevertheless, modifying the side-chain amide, i.e. replacing the pyrrolidine for a *tert*-butyl-group, improved tissue culture potency approximately 2-fold (cf. compounds **24** and **26**). Somewhat surprisingly, unlike compound **25**, incorporation of the backbone ketomethylene isostere into compound **26** improved cell culture potency a further 2-fold. The resultant compound, BILD 1351, has a cell culture potency comparable to that of the larger BILD 1263. The increase in cell culture potency obtained by replacing the pyrrolidine-modified asparagine (compound **24**) with the bis-ketomethylene derivative (BILD 1351) was consistently observed when we incorporated this modification into other ureido-based inhibitors.

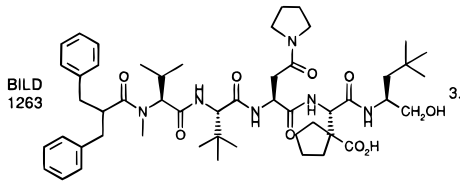
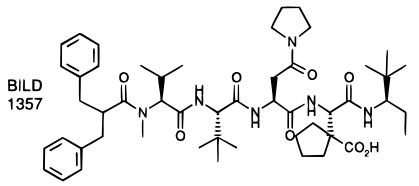
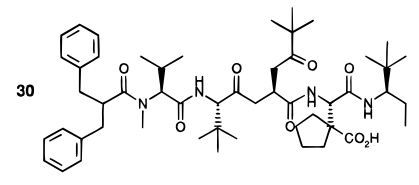
Table 6 uses BILD 1351 as a frame of reference to illustrate the effect on cell culture potency of the previously discussed N- and C-terminal modifications. A comparison between BILD 1351 and compounds **27** and **28** shows the (1-ethylneopentyl)amino C-terminus in BILD 1351 to be superior to either the γ-methylleucinol (**28**) or the unsubstituted neopentylamine (**27**) C-termini. However, a comparison between BILD 1351 and compound **29** shows that the (dimethylcyclohexyl)-amino N-terminus in BILD 1351 provides no benefit in terms of cell culture potency over the (3-propylbutyl)-amino N-terminus. Nevertheless, we maintained a strong interest in the (dimethylcyclohexyl)amino N-terminus after discovering that BILD 1351 appeared to be superior to compound **29** *in vivo* (see next section).

Table 6

Compound *	Cell Culture Assay EC_{50}(μM) HSV-1	HSV-2
BILD 1351 	6 ± 2	7 ± 4
27 	17 ± 7	26 ± 5
28 	26 ± 11	26 ± 5
29 	6 ± 2	5 ± 2

* All compounds have binding assay IC_{50}s below 1 nM

Table 7

Compound *	Cell Culture Assay EC_{50}(μM) ** HSV-1	HSV-2
BILD 1263 	3.1 ± 0.4	4.2 ± 1.0
BILD 1357 	0.8 ± 0.2	1.8 ± 0.5
30 	1.3 ± 0.3	1.6 ± 0.3

* All compounds have binding assay IC_{50}s below 1 nM

** The EC_{50} of acyclovir in this assay is 2 μM

Considering the improvements in cell culture potency obtained from ureido based inhibitors containing the bisketomethylene derivative and the (1-ethylneopentyl)-amino C-terminus, we investigated the effect of these modifications on the antiviral properties of the inhibitor series represented by BILD 1263 (Table 7). As observed previously in the ureido-based inhibitor series, replacement of the C-terminal γ-methylleucinol (BILD 1263) with a 1-ethylneopentylamine (BILD 1357) improved

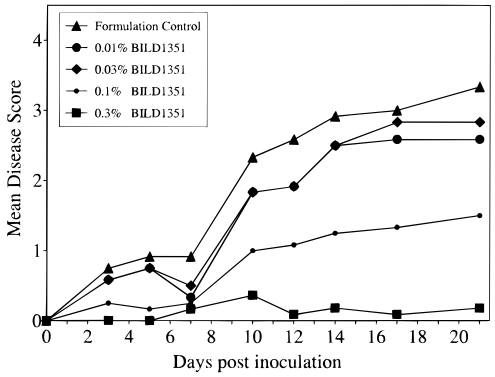


Figure 1. Effect of BILD1351 on HSV-1-induced ketatitis.

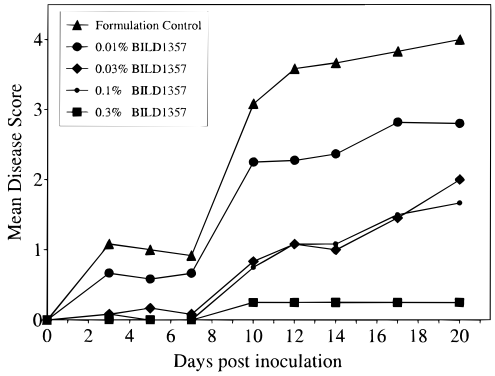


Figure 2. Effect of BILD1357 on HSV-1-induced ketatitis.

cell culture potency. Unfortunately, unlike the ureido-based inhibitor series, replacing the pyrrolidine-modified asparagine residue in BILD 1357 with the bisketomethylene derivative did not improve cell culture potency (see compound **30**). The reason for the latter observation was not immediately clear. Nevertheless, BILD 1357 and compound **30** constituted the most potent inhibitors of HSV RR subunit association made to date.

Inhibitor Potency in a Murine Model of HSV-Induced Keratitis. The potential utility of BILD 1263 as an antiherpetic drug has been highlighted.[6] In addition to cell culture potency comparable to the most widely used antiherpetic drug, acyclovir, BILD 1263 also strongly potentiated the antiviral activity of acyclovir and was effective against acyclovir resistant strains of HSV. Most significantly, BILD 1263 reduced the severity of HSV-1-induced keratitis in a murine ocular model when administered as a 5% ophthalmic cream.[14] We have used this ocular model as a screen for evaluating the relative potency of our newer RR inhibitors, concentrating on testing the most potent inhibitors of HSV replication in cell culture.

BILD 1351 and 1357 proved to be significantly more potent in this model than BILD 1263. As can be seen from Figures 1 and 2, both BILD 1351 and 1357 reduced the severity of HSV-1-induced keratitis in a dose dependent manner. In both cases, disease could be almost completely suppressed (ED_{90}) when the RR inhibitor was administered as a 0.3% cream formulation. We previously showed that BILD 1263 could only come close to this level of disease suppression when administered as a 5% cream formulation.

As mentioned earlier, even though BILD 1351 and compound **29** had similar cell culture potencies, BILD 1351 proved superior in the mouse ocular model.

Compound **29** only managed to achieve the efficacy level of BILD 1351 when administered as a 5−10% cream formulation (data not shown).

Compound **30** was also shown to be a potent inhibitor of HSV replication in cell culture. However, when this compound was applied as a 1% cream formulation in the mouse ocular model, significant hair loss (alopecia) was observed around the eye. Even at higher concentrations of BILD 1351 and 1357, no toxic effect was observed. On this basis compound **30** was not investigated further.

BILD 1351 and 1357 constitute the most potent RR subunit association inhibitors found so far in this *in vivo* model of HSV disease. These results constitute further support for the potential of these peptidomimetic compounds as effective antiherpetic agents. Further pharmacological evaluation of these compounds has commenced.

Conclusion

The compounds disclosed in this paper further highlight the potential of peptidomimetic inhibitors of HSV RR subunit association as potential antiherpetic agents. Structure−activity studies show that inhibitors based on a ureido N-terminus can be as efficacious *in vivo* as the larger (phenylpropionyl)valine based N-terminus. Beneficial modifications that result in improved antiviral potency include a stereochemically defined (2,6-dimethylcyclohexyl)amino N-terminus, a bis-ketomethylene derivative as a replacement for the pyrrolidine modified asparagine residue, and a (1-ethylneopentyl)-amino C-terminus. These three modifications led to the preparation of BILD 1351, our most potent antiherpetic agent containing a ureido N-terminus. The new C-terminal modification allowed the preparation of BILD 1357, a significantly more potent antiviral than our previously published compound, BILD 1263.

Experimental Section

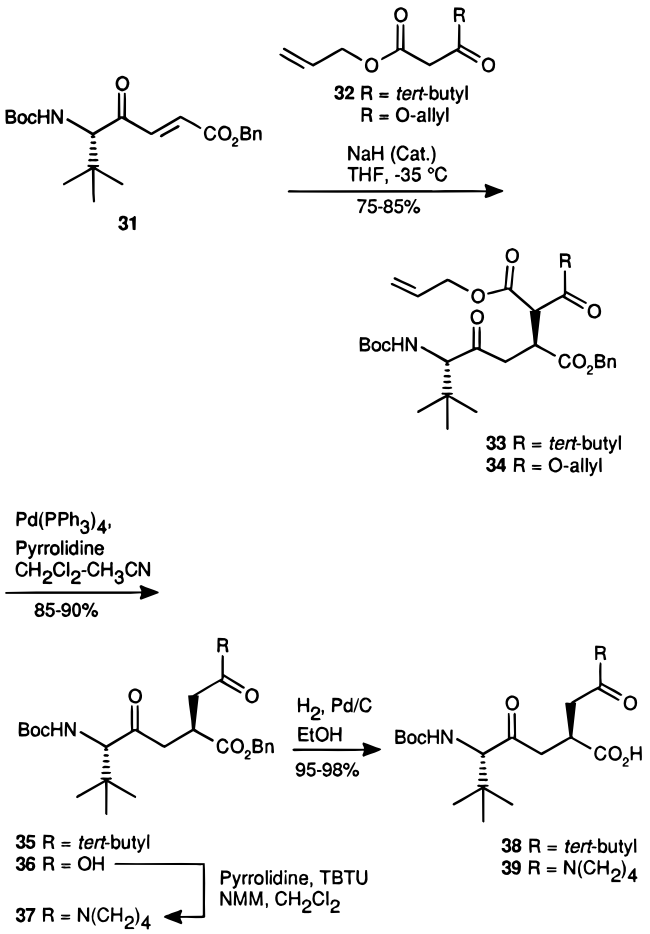
Ribonucleotide Reductase Binding Assay. The inhibitory effect of our compounds in an HSV ribonucleotide reductase radioligand binding assay was measured according to a published protocol.[11] The reported IC_{50} values are the mean of at least four separate determinations, and the standard deviation from the mean is also reported.

HSV Cell Culture Assay. The potencies of our inhibitors against HSV in cell culture were determined by using serum-starved baby hamster kidney (BHK) cells in a colorimetric viral yield assay.[15] The reported EC_{50} values are the mean of at least three separate determinations, and the standard deviation from the mean is also reported. To confirm that the observed antiviral effect was not a consequence of cytotoxicity, the cytotoxic effect of all inhibitors was determined by a tetrazolium salt (MTT) metabolic assay.[16] Typically, selectivity indices (EC_{50} versus CC_{50}) of greater than 15 were observed. BILD 1351, BILD 1357, and compound **29** had selectivity indices of 15−20 while compound **30** showed a lower index of 5−10. All compounds referred to in this paper proved stable under the assay conditions.

Mouse Ocular Model of HSV-Induced Keratitis.[14] Compounds were formulated in a mixture of Eucerin anhydrous, water, and light mineral oil (70:15:15). The desired RR inhibitor was ground with warm mineral oil (37 °C) and then thoroughly mixed with the mixture of Eucerin and water.

Materials. Common amines and *N*-Boc-L-amino acids, *N*-Boc-L-*tert*-leucine, *N*-Boc-L-γ-methylleucine, and L-leucine *O*-benzyl ester pTsOH salt, were obtained from commercial sources. The preparations of Boc-2(*S*)-amino-4-pyrrolidino-4-oxobutanoic acid (pyrrolidine modified asparagine moiety)[8], *N*-Boc-L-γ-methylleucine,[8] and the appropriately protected precursor of cyclopentylaspartic acid[9] have been described in our earlier structure−activity papers. The various stereochemically defined 2,6-dimethylcyclohexylamines were prepared from 2,6-dimethylphenol according to the procedures in refs 17 and 18. The enantioselective synthesis of 2(*R*)- or 2(*S*)-ethyl/methyl-3,3-dimethylpropylamine has been published.[19] The *tert*-butyl ketone amino acid residue found in inhibitors **22** and **26** was prepared according to published procedure.[20]

Scheme 1



The preparation of the ketomethylene dipeptide isosteres found in compound **25** and compounds **27**−**30** and BILD 1351 was based on a diastereoselective synthesis of ketomethylene dipeptide isosteres of the type AAΨ[$COCH_2$]Asp (see Scheme 1).[21] The *tert*-butyl keto ester **32** was prepared as follows. To a solution of lithium bis(trimethylsilyl)amide (1 N, 800 mL) in THF at −78 °C was added dropwise a solution of allyl acetate (39 mL, 0.36 mol) in THF (40 mL). This mixture was stirred at −78 °C under an atmosphere of nitrogen for 1 h, after which time trimethylacetyl chloride (47 mL, 0.38 mol) was added dropwise. After 30 min at −78 °C, hexane (300 mL) followed by 3 N aqueous HCl (600 mL) was added. The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), filtered, and concentrated. This material was distilled bulb to bulb (60 °C air bath, 0.25 Torr) to provide keto ester **32** as a clear colorless liquid (62 g, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.02−5.87 (m, 1 H), 5.35 (br d, J = 17 Hz, 1 H), 5.25 (br d, J = 9.5 Hz, 1 H), 4.63 (br d, J = 5.5 Hz, 2 H), 3.59 (s, 2 H), 1.19 (s, 9 H).

To a solution of keto ester **32** (83.2 g, 0.452 mol) in THF (0.8 L) was added NaH (2.7 g of a 60% oil dispersion, 0.07 mol) over a 15 min period. The reaction mixture was stirred at room temperature under an atmosphere of nitrogen, and the resultant homogeneous solution was cooled to −60 °C. A solution of Michael acceptor **31**[21] (170 g, 0.45 mol) in THF (0.5 L) was added over a period of 45 min, and the reaction mixture was stirred at −60 °C for 5 h. A 10% aqueous solution of citric

acid (500 mL) was added, and the mixture was allowed to warm to room temperature. The mixture was extracted with Et_2O (1 L). The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and concentrated to afford crude **33** as an orange oil (250 g). This material was used without further purification in the next reaction. To a solution of tetrakis(triphenylphosphine)-palladium(0) (2.60 g, 2.25 mmol, 0.5% molar) in CH_2Cl_2 (250 mL) and CH_3CN (250 mL) at 0 °C was added pyrrolidine (56 mL, 0.54 mol). The mixture was allowed to warm to room temperature, and a solution of **33** (250 g, 0.45 mol) in CH_2Cl_2 (200 mL) and CH_3CN (200 mL) was added. After 3 h, the mixture was concentrated to yield an orange oil. This material was dissolved in Et_2O−hexane (1:1, 1 L). The resultant solution was washed with a 10% aqueous citric acid, 10% aqueous $NaHCO_3$, and brine, dried ($MgSO_4$), filtered, and concentrated to give an orange oil (203 g). This material could be used in the next reaction without further purification or it could be purified by silica gel flash chromatography (elution with hexane−EtOAc, 9:1) to give compound **35** as a colorless oil. The diastereoisomeric purity was assessed to be >35:1 by NMR: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38−7.28 (m, 5 H), 5.10 (s, 2 H), 5.07 (br d, J = 9 Hz, 1 H), 4.08 (d, J = 9 Hz, 1 H), 3.38−3.31 (m, 1 H), 3.09 (dd, J = 19, 6 Hz, 1 H), 2.94 (dd, J = 18.5, 6 Hz, 1 H), 2.82 (dd, J = 18.5, 6 Hz, 1 H), 2.77 (dd, J = 19, 6 Hz, 1 H), 1.42 (s, 9 H), 1.10 (s, 9 H), 0.95 (s, 9 H).

To a solution of crude compound **35** (171 g, ∼0.36 mol) in EtOH (1.4 L) was added 10% Pd/C (10 g). The resultant mixture was stirred vigorously under 1 atm of hydrogen for 5 h. Thereafter, the reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure, and the residue was dissolved in saturated aqueous Na_2CO_3. The aqueous solution was washed with hexane−Et_2O (8:2), rendered acidic with citric acid and extracted with EtOAc. The organic phase was dried ($MgSO_4$) and concentrated. The resultant orange residue was dissolved in Et_2O and the solution was passed through a silica gel pad (12 × 12 cm). Concentration provided compound **38** as a white solid (117 g, 84% yield): mp 62−65 °C; ^{1}H NMR ($CDCl_3$) δ 5.18 (d, J = 9 Hz, 1 H), 4.09 (d, J = 9 Hz, 1 H), 3.35−3.29 (m, 1 H), 3.09 (dd, J = 19, 6.5 Hz, 1 H), 2.94 (dd, J = 18.5, 6.5 Hz, 1 H), 2.83 (dd, J = 18.5, 6.5 Hz, 1 H), 2.78 (dd, 19, 6.5 Hz, 1 H), 1.43 (s, 9 H), 1.14 (s, 9 H), 0.96 (s, 9 H).

Compound **36** could be prepared as described above for compound **35** by using diallyl malonate. Decarboxylation following deallylation of the diallyl malonate derivative **34** required heating of the crude diacid in xylene. Compound **36** was obtained as a clear gum: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.38−7.21 (m, 5 H), 7.27 (d, J = 8 Hz, 1 H), 5.06 (s, 2 H), 3.79 (d, J = 8 Hz, 1 H), 3.14−3.08 (m, 1 H), 2.92 (dd, J = 18.5, 6 Hz, 1 H), 2.79 (dd, J = 18.5, 6 Hz, 1 H), 2.54 (dd, J = 17, 7 Hz, 1 H), 2.44 (dd, J = 17, 6 Hz, 1 H), 1.38 (s, 9 H), 0.89 (s, 9 H). Compound **36** could be coupled with pyrrolidine according to the general coupling procedure in the inhibitor synthesis section. Compound **37** was obtained as a clear gum: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29−7.22 (m, 5 H), 5.08 (d, J = 8.5 Hz, 1 H), 5.05 (s, 2 H), 4.01 (d, J = 8.5 Hz, 1 H), 3.40−3.20 (m, 5 H), 3.10 (dd, J = 18.5, 7 Hz, 1 H), 2.88 (dd, J = 18.5, 5 Hz, 1 H), 2.63 (dd, J = 16, 5.5 Hz, 1 H), 2.48 (dd, J = 16, 7 Hz, 1 H), 1.87−1.72 (m, 4 H), 1.35 (s, 9 H), 0.88 (s, 9 H). Compound **39** was obtained as a clear gum from **37** using the hydrogenolysis procedure described for compound **38**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.22 (d, J = 8.5 Hz, 1 H), 3.80 (d, J = 8.5 Hz, 1 H), 3.38−3.21 (m, 4 H), 3.04−2.98 (m, 1 H), 2.82 (dd, J = 18.5, 6 Hz, 1 H), 2.72 (dd, J = 18.5, 6 Hz, 1 H), 2.52 (dd, J = 16.5, 7 Hz, 1 H), 2.38 (dd, J = 16.5, 6.5 Hz, 1 H), 1.88−1.72 (m, 4 H), 1.39 (s, 9 H), 0.91 (s, 9 H).

Inhibitor Synthesis. All inhibitors were prepared by sequentially coupling *N*-Boc-amino acid derivatives (azide derivative for cyclopentylaspartic acid, see ref 9) from C- to N-terminus by using benzotriazol-1-yl 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as the coupling agent. Removal of the *N*-Boc protective group was effected with 4 N HCl in dioxane (azide derivative of cyclopentylaspartic acid reduced to amine with $SnCl_2$ in methanol). Representative procedures can be found in reference 9.

The various N-terminal alkylureido functionalities were introduced as follows. To a solution of the appropriate peptide hydrochloride salt (0.5 mmol) in dry dichloromethane (5 mL) was added *N*-methylmorpholine (1.5 mmol) and the relevant[22] isocyanate (1 mmol, usually neat). The reaction mixture was stirred at room temperature for 16 h, after which time the volatiles were removed and the residue was partitioned between EtOAc and 0.1 M aqueous HCl. The organic phase was washed with 5% aqueous sodium bicarbonate and brine, dried ($MgSO_4$), filtered, and concentrated. The resultant crude product was purified by silica gel flash chromatography to provide the desired ureido derivatives usually as white foams (64−76% yield).

The last reaction involved in inhibitor preparation involved removal of the benzyl ester protective group(s) by catalytic hydrogenolysis (10 mol % of 10% Pd/C in methanol under 1 atm of H_2 for 3 h). The resultant inhibitor was often obtained in greater than 95% purity (HPLC and NMR), but when necessary it was purified by preparative HPLC on a C18 reverse-phase column (Vydac, 15 μm particle size) eluting with 0.06% TFA in water−0.06% TFA in acetonitrile gradients.

Inhibitor Characterization and Purity. All inhibitors showed satisfactory ^{1}H NMR spectra (400 MHz), FAB mass spectra ($M^+ + H$) and/or ($M^+ + Na$), and HPLC purity in two solvent systems (>95%). All inhibitors not containing the ketomethylene isostere fragment showed satisfactory amino acid analysis including peptide recovery. Satisfactory elemental analysis was obtained for BILD 1351 and BILD 1357. An X-ray structure of compound BILD 1351 was obtained, providing proof of the assigned stereochemistries for all asymmetric centers.

Acknowledgment. We are grateful to M. Liuzzi and E. Scouten for determining the IC_{50}s of our inhibitors in the binding assay. We are also grateful to C. Bousquet and N. Dansereau for determining EC_{50}s of our inhibitors in cell culture. We are also grateful to L. Tong for the X-ray structure of BILD 1351.

Supporting Information Available: Full tabulation of ^{1}H NMR, FAB mass spectra, amino acid analysis, elemental analysis, HPLC purity data for new inhibitors and coordinates for the X-ray structure of BILD 1351 (21 pages). Ordering information is given on any current masthead page.

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(12) This observation has also been made for γ-methylleucine versus leucine (ref 9) and γ-methylleucinol versus leucinol (ref 8).

(13) An approximately one log unit increase in log *K*′w was observed between inhibitors **24** and **26** and **25** and BILD 1351 (HPLC solvent: 60−75% MeOH−aqueous Na_2HPO_4, 20 mM, pH 7.4).

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