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Synthesis and SAR studies on azetidine-containing dipeptides as HCMV inhibitors

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ABSTRACT

SAR studies on an azetidine-containing dipeptide prototype inhibitor of HCMV are described. Three series of structurally modified analogues, involving substitutions at the N- and C-terminus, and at the C-terminal side-chain were synthesized and evaluated for antiviral activity. Aliphatic or no substituents at the C-carboxamide group, an aliphatic C-terminal side-chain, as well as a benzyloxycarbonyl moiety at the N-terminus were absolute requirements for anti-HCMV activity. The conformational restriction induced by the 2-azetidine residue into the dipeptide derivatives, identified by 1H NMR as a γ-type reverse turn, seems to have influence on the activity of these molecules.

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1. Introduction

Human cytomegalovirus (HCMV) is a prevalent β-herpesvirus that can cause serious, life-threatening diseases in immunologically immature or immunocompromised individuals, like neonates, AIDS patients, and transplant recipients. Moreover, infection with HCMV is suggested to be associated with certain vascular diseases (atherosclerosis, restenosis, etc.).

The current antiviral agents approved for HCMV infection, ganciclovir, acyclovir, their Val pro-drugs valganciclovir and valaciclovir, cidofovir, foscarnet and the antisense RNA fomivirsen, as HCMV inhibitors have been described. Most of them include an activated carbonyl group or are mechanism-based inhibitors that covalently interact with the Ser residue within the enzyme active site. Among the latter, we and others have discovered simple and small β-lactam derivatives with anti-HCMV activity. In our case, a simple deletion of the carbonyl group of the β-lactam ring resulted in the corresponding azetidine derivatives, which behave as non-covalent inhibitors of HCMV replication. Preliminary structure–activity relationship studies suggested the importance of a certain hydrophobic environment by the carbamateylate in position 2 of the azetidine ring. Moreover, within this series, some C-terminal carboxamide derivatives showed restricted rotation around the N1–CO bond, due to the stabilization of a γ-turn-like folded conformation. To investigate further the structural and conformational issues within these azetidine derivatives and to determine their interest as antiviral agents, we initiated a program directed to the modification of compound 1, selected as a prototype (Fig. 1). To this end, diverse substituents have been built-in the C-terminal carboxamide moiety (A), amino acids with side-chains of different ligands for the chemokine receptor US28, thus also acting at an early stage of the replication cycle.

Quite recently, some γ-sultone derivatives with anti-HCMV activity were shown to keep full antiviral sensitivity against a panel of mutant HCMV strains, selected for resistance against approved drugs, and accordingly suggesting a new mechanism of action, which is still unclear.

The HCMV protease, essential for capsid assembly and viral maturation, is being considered an attractive target for anti-HCMV chemotherapy, and several structurally diverse inhibitors have been described. Most of them include an activated carbonyl group or are mechanism-based inhibitors that covalently interact with the Ser residue within the enzyme active site. Among the latter, we and others have discovered simple and small β-lactam derivatives with anti-HCMV activity. In our case, a simple deletion of the carbonyl group of the β-lactam ring resulted in the corresponding azetidine derivatives, which behave as non-covalent inhibitors of HCMV replication. Preliminary structure–activity relationship studies suggested the importance of a certain hydrophobic environment by the carbamateylate in position 2 of the azetidine ring. Moreover, within this series, some C-terminal carboxamide derivatives showed restricted rotation around the N1–CO bond, due to the stabilization of a γ-turn-like folded conformation. To investigate further the structural and conformational issues within these azetidine derivatives and to determine their interest as antiviral agents, we initiated a program directed to the modification of compound 1, selected as a prototype (Fig. 1). To this end, diverse substituents have been built-in the C-terminal carboxamide moiety (A), amino acids with side-chains of different ligands for the chemokine receptor US28, thus also acting at an early stage of the replication cycle.
character were selected as C-terminal residues (B), and the N-terminal benzylxycarbonyl group was replaced by different urethane, acyl and urea moieties containing aromatic groups (C).

This paper deals with the solution/solid-phase synthesis, conformational analysis by NMR, and biological assays of the compounds resulting from the three indicated series A–C of azetidine-containing dipeptides.

2. Results and discussion

2.1. Synthesis

To increase the hydrophobic character of compound 1, different alkyl groups were incorporated at the C-terminal carboxamide to generate derivatives 4 (Series A). The preparation of these compounds was achieved by coupling the 2-carboxy azetidine 2 with the corresponding i-alanyl carboxamides 3 under standard solution methodologies (Scheme 1). All final compounds 4a–e were obtained in good yield as mixtures of two diastereoisomers that could be chromatographically resolved. Since the starting azetidine was a ~2:1 S:S enantiomeric mixture, the major components of these mixtures were assigned as the S,S-diastereoisomers.

To understand the role of the C-terminal amino acid side-chain, d-Ala-, Phe-, Val-, Leu-, Lys- and Glu-bearing derivatives 7a–e were designed (series B). Solid-phase synthetic methodologies, employing a Rink-amide polystyrene resin as insoluble matrix, were used for the preparation of these compounds (Scheme 2). After removal of the Fmoc-protecting group from the commercial resin, the corresponding Fmoc-Xaa-OH amino acids were coupled, using DIC/HOBt, to afford intermediates 5a–f. A similar procedure was followed for the subsequent incorporation of the 1-benzylxycarbonyl azetidine 2. The resulting resin-bound azetidine-containing dipeptides 6a–f were finally detached from the polymeric support by treatment with a 95:4:1 TFA/H2O/TIPS cleavage cocktail. As for the previous series, compounds 7 were obtained in excellent yields as mixtures of two diastereoisomers that were separated by column chromatography, and configurationally assigned as previously indicated for analogues 4.

Taking into account the inactivity of the N-methoxycarbonyl-substituted analogue of 1,14b which suggested a role for the aromatic phenyl ring of the Z-group, different Ar-containing urethane, acyl and urea groups were envisaged as substituents at the N1 position of the azetidine moiety (series C). The good results in the synthesis of compounds 7 prompted us to apply a similar solid-phase approach for the preparation of the third series of derivatives (Scheme 3). In this case, the Fmoc-L-Ala resin 5g was deprotected and coupled to the Fmoc-azetidine derivative 9. Removal of the Fmoc-protecting group from 9 followed by reaction with different chloroformates or acyl- or sulfonyl-chlorides in the presence of propylene oxide gave to derivatives 10b–g. After cleavage, alternatively, the treatment with isocyanates, followed by exposure to the cleavage cocktail, allowed the preparation of urea analogues 10h–l. In all these cases, only the major S,S-diastereoisomer of the mixture was isolated in enough purity for biological assays. The deficient coupling of azetidine derivative 8 could be responsible for the formation of the complex reaction crudes, making the purification step more difficult.

All prepared compounds were characterised by standard analytical and spectroscopic methods. The 1H NMR spectra always showed cis/trans rotamer around the CON bond, fluctuating...
between 10% and 20% of cis conformation in CDCl₃ and increasing up to 40% in DMSO-d₆.

2.2. Conformational analysis by ¹H NMR

In our previous work on anti-HCMV N-benzylxoycarbonylazetidines, we found that some active 2-carbamoyl derivatives had low conformational flexibility, due to the formation of a reverse γ-turn, stabilized through an intramolecular hydrogen bond between the Z carbonyl group and the NH proton of the 2-carboxamide.¹⁴ Recent studies on 2-alkyl-2-carboxy azetidine-containing model peptides confirmed that these non-proteinogenic amino acids are valuable γ-turn inducers.¹⁶ To ascertain whether this particular folded conformation could play or not a role in the antiviral activity of the new prepared compounds, their chemical shifts and temperature coefficients for amide protons were evaluated.

In CDCl₃, the chemical shift values for the α-NH amide protons in the major trans-rotamer were always >7 ppm, and the variation when changing to DMSO-d₆ was very small (0.01–0.3 ppm in most cases), suggesting the participation of these protons in intramolecular stable hydrogen bonds that protect them from solvent influence.¹⁷ On the contrary, the C-terminal CONH₂ or CONHR amide protons showed chemical shifts in the range of 5.27–6.82 ppm in CDCl₃, while the change of solvent resulted in an important fluctuation of δ values, indicative of solvent exposure (normally >1 ppm, see Table 15, Supplementary data).

The variation of amide proton chemical shifts with the temperature was measured in DMSO-d₆ for all compounds 4, 7 and 10, and the calculated temperature coefficients are recorded in Table 1. It is established that, in small peptides, values below 3 ppb/K (in absolute value) are indicative of solvent-protected NH, probably implicated in a hydrogen bond, while values above 4 ppb/K indicated accessibility to solvent or non-hydrogen-bonded states, and those between 3 and 4 ppb/K are in the range of uncertainty.¹⁸ As shown in the table, most compounds showed Δα/ΔT values for the α-NH amide proton <3 ppb/K, while for a small number of them the values were not conclusive. These data point towards the stabilization of a γ-turn structure through the formation of an intramolecular hydrogen bond implicating the α-NH amide proton. In contrast, the temperature coefficients for the C-terminal amide protons are large enough, indicating total accessibility to the solvent. The only exception was compound (R)-4b, for which the Δα/ΔT values for both amide protons suggested either the coexistence of two conformational states, β- and γ-turn or a single double-turned conformation.

2.3. Antiviral activity

The azetidine-derived dipeptides of the three series, A–C, were evaluated for their ability to inhibit the replication of HCMV in vitro.¹⁹ The results were compared to those of the reference compound 1 and the commercial drug ganciclovir (Tables 2–4).

As shown in Table 2, derivatives (S,S)-4a, (R,S)-4a and (S,S)-4c, with terr-butyl and iso-butyl groups at the C-terminal amide, retain the anti-HCMV activity with EC₅₀ values comparable to model dipeptide 1. Benzyl- and cyclohexyl-substituted analogues were
Table 2
Activity of 1-Z-Azf-Ala- NR^2 derivatives against human cytomegalovirus (HCMV)

| Compd  | R^1, R^2   | EC_{50} (µM) | Cytotoxicity (µM) |
|--------|------------|--------------|-------------------|
|        | HCMV^a     | MCC^b        | CC_{50}^c         |
| (S,S)-4a | H, CH(CH_3)_2 | 45 >100       | >100 >100        |
| (R,S)-4a | H, CH(CH_3)_2 | 49 >100       | >100 >100        |
| (S,S)-4b | H, CH(CH_3)_2 | >20 >100      | >100 >100        |
| (R,S)-4b | H, CH(CH_3)_2 | >4 >20        | >100 >100        |
| (S,S)-4c | H, CH(CH(CH_3)_2 | >100 >100     | >100 >100        |
| (R,S)-4c | H, CH(CH(CH_3)_2 | >100 >100     | >100 >100        |
| (S,S)-4d | H, Chx      | >100 >100     | >100 >100        |
| (R,S)-4d | H, Chx      | >20 100       | 100 46           |
| (S,S)-4e | CH_2, CH_3  | >100 >100     | >100 >100        |
| (R,S)-4e | CH_2, CH_3  | >100 >100     | >100 >100        |
| (S,S)-4f | H, H,C      | 32 >50        | 50 33            |
| (R,S)-4f | H, H,C      | 32 >50        | 50 33            |
| 11     | Z-Phe-Ala-NH_2 | >100 >100     | >100 >100        |
| 12     | Z-Phe-Ala-NH^tBu | >100 >100     | >100 >100        |
| GCV    | 7.0 >1575   | 580          |

^a Effective concentration required to reduce virus plaque formation by 50% (Davis strain). Virus input was 100 plaque forming units (PFU).

^b Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

^c Cytotoxic concentration required to reduce cell growth by 50%.

Table 3
Activity of 1-Z-Azf-Xaa-NH_2 derivatives against human cytomegalovirus (HCMV)

| Compd  | R^3   | EC_{50} (µM) | Cytotoxicity (µM) |
|--------|-------|--------------|-------------------|
|        | HCMV^a | MCC^b        | CC_{50}^c         |
| (S,R)-7a | CH_3  | 63 >100       | >100 36           |
| (R,R)-7a | CH_3  | >100 >100     | >100 >100         |
| (S,S)-7b | CH_2,Ph | >20 100       | 100 37           |
| (R,S)-7b | CH_2,Ph | >20 100       | 100 37           |
| (S,S)-7c | CH(CH_2)_2 | 76 >100      | 100 85           |
| (R,S)-7c | CH(CH_2)_2 | >100 >100   | >100 >100        |
| (S,S)-7d | CH(CH(CH_3)_2 | 20 100       | 100 50           |
| (R,S)-7d | CH(CH(CH(CH_3)_2 | 45 >100      | >100 66           |
| 7e     | (CH_2)_3NH_2 | >100 >100     | >100 >100        |
| 7f     | (CH_2)_3COH  | >100 >100     | >100 >100        |
| (S,S)-1 | CH_3 | 32 >50        | 50 50            |
| GCV    | 7.0 >1575   | 580          |

^a, b and c as defined in Table 2.

As indicated in the previous section, the described azetidine dipeptides adopt a γ-turn-like conformation, centred at the azetidine residue. To explore to which extent this structural characteristic could be important for HCMV antiviral activity, dipeptides Z-Phe-Ala-NH_2 (11) and Z-Phe-Ala-NH^tBu (12), non-constrained analogues of active compounds 1 and 4a, respectively, were synthesized and evaluated. All the amide protons of peptides 11 and 12 showed temperature coefficients higher than 4.5 ppb/K, in absolute value, indicating random coil conformations, and therefore higher flexibility than azetidine-containing analogues. Both dipeptides were unable to inhibit the replication of HCMV in cell culture (Table 2), suggesting a key function of the azetidine ring in the observed antiviral activity. A direct role, in which the methylene groups of the four-member ring interact with the target, or an indirect function to spatially pre-organise the molecule to facilitate the interaction through other functionalities, could be envisaged.
In series B (Table 3), only compounds (SR)-7a, (SS)-7c and (RS)-7d, derived from o-Ala, -Val and -Leu, respectively, maintain measurable anti-HCMV activity, while the Phe, Lys, and Glu analogues were inactive. Again, a preference for aliphatic groups at the C-terminal residue is observed. However, neither an increase of the aliphatic side-chain volume (Val or Leu vs Ala) nor a change in the configuration of the Ala residue succeeded in improving the activity of prototype 1. Active compounds in the B series are—as a rule—also somewhat cytotoxic, specially the o-Ala- and Phe-containing analogues.

As shown in Table 4, most prepared derivatives in compound series C were totally inactive at 100 μM, except for compound (SS)-10e, bearing a 1-naphthylcarbamate group. This group can be considered as a conformationally restricted analogue of the benzzyloxycarbonyl substituent present in prototype 1. These results suggest that the Z group plays a key role in the activity of these azetidine-derived compounds. In fact, small changes, such as the incorporation of different types of substituents at the aromatic ring (10b, 10c), the shortening of the benzyl group to phenyl (10d), and the replacement of the urethane oxygen atom by CH2 or NH (10g, 10h, 10i), led to inactive analogues. Compound 10a, being inactive against HCMV, was the most cytotoxic/cytostatic compound among the three series of azetidine derivatives (Table 3).

To assess HCMV selectivity, all compounds were also evaluated for antiviral activity against a wide panel of DNA and RNA viruses. Some active derivatives against HCMV also inhibited the replication of varicella-zoster virus (VZV, Table 2S within Supplementary data), while they were inactive against a wide variety of other DNA and RNA viruses at subtoxic concentrations (20–100 μM). Dual anti-VZV and anti-HCMV activity was also described for non-nucleoside 4-benzoxy-γ-sultones and bicyclic furanopyrimidine-derived nucleosides.19,20

3. Conclusion

Structure–activity studies on a new family of azetidine-containing dipeptide inhibitors of HCMV are described. Compounds modified at the C-terminal residue (carboxamide and amino acid side-chain) and at the N-terminus were easily synthesized by application of both solid-phase and solution methodologies. The results of the biological assays indicated quite strict structural requirements for activity. Thus, only derivatives with aliphatic groups at the C-terminal residue, either at the carboxamide moiety or at the side-chain showed significant inhibitory activity against HCMV replication in cell cultures. Additionally, at the C-carboxamide group, the presence of a hydrogen atom is an absolute requirement. Substitutions at N-terminus recommended the initial benzzyloxycarbonyl (Z) group as the best choice at this position. In addition, the conformational restriction induced by the reverse turn (γ-type) seems important for activity, since more flexible dipeptide analogues were totally inactive. Further SAR studies are needed to improve the activity of the present series of inhibitors.

4. Experimental

For details on general methods and complete 1H, 13C and MS characterisation of all intermediates and final compounds, see the Supplementary data. Azetidine 2 was synthesized according to the previously reported method.14b

4.1. (2R,5)-2-Benzyl-1-(9-fluorenylmethoxy)carbonyl-2-carboxyazetidine (8)

A solution of (2R,5)-2-benzyl-2-methoxycarbonylazetidine3a (1.57 g, 7.64 mmol) in MeOH (20 mL) was treated with 2 N NaOH (19.1 mL, 9.55 mmol). The reaction was stirred at rt for 24 h. The solvent was removed and the residue dissolved in water and washed with EtOAc. The aqueous layer was lyophilised and the resulting residue was dissolved in a mixture of Na2CO3 (10%) and dioxane (20 mL/12 mL). The reaction was cooled at 0 °C and a solution of Cl-Fmoc (1.97 g, 7.64 mmol) in dioxane (12 mL) was added. After 1 h, the temperature was allowed to raise and the reaction was stirred for 3 h. Then, water was added and the mixture was washed with EtO. The organic layer was acidified with 1 M HCl up to pH 2 and extracted with EtOAc. The organic layer was washed with brine, dried over Na2SO4 and the solvent removed under vacuum. The residue was purified by flash chromatography employing a mixture of EtOAc/CH2Cl2 (1:7) to afford 1.89 g (50%) of the title azetidine 8 as a solid.

4.2. Synthesis of azetidine-derived dipeptides

4.2.1. Series A

General procedure for the coupling reaction: A solution of the corresponding H-Ala-NR2 trifluoroacetate (0.36 mmol) in anhydrous THF, was treated with azetidine 2 (58 mg, 0.18 mmol), BOP (0.16 g, 0.36 mmol) and Et3N (0.1 mL, 0.72 mmol). The reaction was stirred overnight at rt. The solvent was evaporated and the resulting residue was dissolved in EtOAc and washed with citric acid (10%), NaHCO3 (10%) and brine. The organic layer was washed with Na2SO4 and evaporated affording a residue, which was purified by flash chromatography (EtOAc/Et2O/hexane, 1:1:1).

4.2.1.1. (2S,5)-2-Benzyl-1-benzyloxycarbonyl-2-[1-(tert-butylcarbonyl)-ethylcarbamoyl]azetidine, (SS)-4a

Syrup. Yield: 58%. [x]D = 7.3 (c 0.49, CHCl3). HPLC: Rf = 19.79 min (A/B = 50:50). 1H NMR (DMSO-d6), rotamers ratio: 5.2:1. Major rotamer δ: 8.20 (br d, 1H, J = 7.7, 1H-NH), 7.79 (br d, 1H, J = 7.3, NH-Bu), 7.45–7.13 (m, 10H, Ph), 5.24 (d, 1H, J = 12.4, CH2-Z), 5.04 (d, 1H, J = 12.4, CH2-Z), 4.29 (m, 1H, 1H-CH2), 3.53 (m, 1H, 1H-4), 3.31 (d, 1H, J = 13.9, 2-CH2), 3.03 (d, 1H, J = 13.9, 2-CH2), 2.88 (dd, 1H, J = 8.1 and 16.5, 4-H), 2.32 (m, 1H, 3-H), 2.10 (m, 1H, 3-H), 1.27 (s, 9H, Bu), 1.20 (d, 3H, J = 6.9, 1-CH3). MS: 452 [M+1]+, 474.2 [M+Na]+. Anal. Calc. for C30H32N2O4: C, 75.78; H, 5.73; N, 3.42.

4.2.1.2. (2R,5)-2-Benzyl-1-benzyloxycarbonyl-2-[1-(tert-butylcarbonyl)-ethylcarbamoyl]azetidine, (RS)-4a

Syrup. Yield: 25%. [x]D = –47.5 (c 0.61, CHCl3). HPLC: Rf = 17.60 min (A/B = 50:50). 1H NMR (DMSO-d6), rotamers ratio: 4.6:1. Major rotamer δ: 8.14 (d, 1H, J = 7.7, 1H-NH), 7.81 (br d, 1H, J = 7.6, NH-Bu), 7.46–7.13 (m, 10H, Ph), 5.23 (d, 1H, J = 12.4, CH2-Z), 5.06 (d, 1H, J = 12.4, CH2-Z), 4.29 (m, 1H, 1H-CH2), 3.50 (m, 1H, 1H-4), 3.36 (d, 1H, J = 13.9, 2-CH2), 2.98 (d, 1H, J = 13.9, 2-CH2), 2.79 (m, 1H, 1H-4), 2.30 (m, 1H, 3-H), 2.11 (m, 1H, 3-H), 1.25 (s, 9H, Bu), 1.23 (d, 3H, J = 7.3, 1-CH3). MS: 452.2 [M+1]+, 474.2 [M+Na]+. Anal. Calc. for C30H32N2O4: C, 69.16; H, 7.37; N, 9.31. Found: C, 68.87; H, 7.61; N, 9.29.

4.2.1.3. (2S,5)-2-Benzyl-1-benzyloxycarbonyl-2-[1-(tert-isobutyloxycarbonyl)-ethylcarbamoyl]azetidine, (SS)-4c

Foam. Yield: 60%. [x]D = +14.7 (c 0.5, CHCl3). HPLC: Rf = 22.29 min (A/B = 47:53). 1H NM (DMSO-d6), rotamers ratio: 2.8:1. Major rotamer δ: 8.29 (d, 1H, J = 7.3, 1H-NH), 7.85 (m, 1H, NH-Bu), 7.44–7.10 (m, 10H, Ph), 5.25 (d, 1H, J = 12.4, CH2-Z), 5.04 (d, 1H, J = 12.4, CH2-Z), 4.34 (m, 1H, 1H-4), 3.54 (m, 1H, 1H-4), 3.31 (d,
1H, J = 13.9, 2-CH$_2$), 3.01 (d, 1H, J = 13.9, 2-CH$_2$), 2.95 (m, 1H, 4-H), 2.84 (m, 2H, CH$_2$-Bu), 2.32 (m, 3H, 3'-H), 2.01 (m, 1H, 3'-H), 1.72 (m, 1H, CH$_2$-Bu), 1.26 (d, 3H, J = 7.1, 1'-CH$_3$), 0.85 (d, 6H, J = 6.6, CH$_2$-Bu). MS: 452.3 [M+1]$^+$, 474.3 [M+Na]$^+$. Anal. Calcld for C$_{26}$H$_{33}$N$_2$O$_4$: C, 69.16; H, 7.37; N, 9.31. Found: C, 68.91; H, 7.29; N, 9.15.

4.2.2. Series B and C

4.2.2.1. (2S$_{	ext{a}}$)-5-(benzylthio)-1-methyl-2-(1'-carbamoyl)-1-phenylcarbonylazetidines, (S$_{a}$)-7a 

Fmoc group from resin 0

4.2.2.2. (2R,-1'S)-5-(benzylthio)-1-methyl-2-(1'-carbamoyl)-1-phenylcarbonylazetidines, (R$_{5}$)-7c 

Synthesis of N-substituted derivatives of series C: After removal of Fmoc group from resin 9, propylene oxide (15 equiv, except for the reaction with isocyanate) was added. The reaction was cooled at 0°C and 10 equiv of the corresponding isocyanate, chlorofomate, acetyl- or sulfon chloride were added. After 18 h of slow stirring at room temperature, the excess of reagents was eliminated by successive washes with DMF/DCM/DMF/DCM (4 × 0.5 min) to afford intermediates 5a–g.

The efficiency of the coupling reactions was monitored by the Kaiser’s test. A similar procedure was followed for the subsequent incorporation of azetidines 2 and 8.

4.2.2.4. (2S$_{5}$,1'S)-2-Benzyl-2-[(1'-carbamoyl)ethyl]carbamoyl-1-(1-naphthoxy)carbonylazetidine, (S$_{5}$)-10e

Syrup. Acetone/CH$_2$Cl$_2$, 1:6. Yield: 52%. [x]$_D$ = +18.1 (c 0.8, CHCl$_3$). H NMR (DMF-d$_6$): 14.3 (br s, 1H, CONH$_2$), 7.62–7.35 (m, 13H, Ar, CONH$_2$), 7.31 (br s, 1H, CONH$_2$), 4.32 (m, 1H, 1'-H), 3.64 (m, 1H, 4-H), 3.27 (d, 1H, J = 13.8, 2-CH$_2$), 3.18 (d, 1H, J = 13.8, 2-CH$_2$), 2.98 (m, 1H, 4-H), 2.33 (m, 1H, 3'-H), 2.28 (m, 1H, 3'-H), 1.28 (d, 3H, J = 7.6, 1'-CH$_3$). MS: 343.2 [M+1]$^+$. Anal. Calcld for C$_{24}$H$_{24}$N$_2$O$_4$: C, 69.59; H, 5.84; N, 9.74. Found: C, 69.66; H, 5.88; N, 9.68.

4.2.2.5. (2S$_{5}$,1'S)-2-Benzyl-2-[(1'-carbamoyl)ethyl]carbamoyl-1-(2-naphthoxy)sulfonazetidine, (S$_{5}$)-10f

Solid. Acetone/CH$_2$Cl$_2$, 1:6. Yield: 42%. Mp: 154–156°C. [x]$_D$ = +14.7 (c 0.8, CHCl$_3$). H NMR (DMF-d$_6$): 14.3 (br s, 1H, CONH$_2$), 7.72–7.18 (m, 10H, Ph), 7.05 (br s, 1H, CONH$_2$), 4.26 (m, 1H, 1'-H), 3.63 (m, 1H, 4-H), 3.33 (d, 1H, J = 13.6, 2-CH$_2$), 2.98 (d, 1H, J = 13.8, 2-CH$_2$), 2.98 (m, 1H, 4-H), 2.82 (m, 2H, CH$_2$CH$_2$Ph), 2.34 (m, 2H, CH$_2$CH$_2$Ph), 2.24 (m, 1H, 3'-H), 2.07 (m, 1H, 3'-H), 1.36 (d, 3H, J = 7.3, 1'-CH$_3$). MS: 452.2 [M+1]$^+$. Anal. Calcld for C$_{26}$H$_{26}$N$_2$O$_4$: C, 63.84; H, 5.58; N, 9.31. Found: C, 63.88; H, 5.61; N, 9.28.

4.2.2.6. (2S$_{5}$,1'S)-2-Benzyl-2-[(1'-carbamoyl)ethyl]carbamoyl-1-phenetylcarbonylazetidines, (S$_{5}$)-10g

Foam. 5–17% Acetone in CH$_2$Cl$_2$, Yield: 70%. [x]$_D$ = +20.0 (c 1.3, CHCl$_3$). H NMR (DMF-d$_6$): 14.3 (br s, 1H, CONH$_2$), 7.41–7.13 (m, 11H, Ph, CONH$_2$), 7.05 (br s, 1H, CONH$_2$), 5.23 (d, 1H, J = 12.5, CH$_2$-Z), 5.03 (d, 1H, J = 12.5, CH$_2$-Z), 4.28 (m, 1H, 1'-H), 3.49 (m, 1H, 4-H), 3.37 (d, 1H, J = 13.8, 2-CH$_2$), 2.99 (d, 1H, J = 13.8, 2-CH$_2$), 2.79 (m, 1H, 4-H), 2.30 (m, 1H, 3'-H), 2.11 (m, 1H, 3'-H), 1.18 (d, 3H, J = 6.7, 1'-CH$_3$). MS: 396.1 [M+1]$^+$. Anal. Calcld for C$_{25}$H$_{25}$N$_3$O$_4$: C, 66.73; H, 6.37; N, 10.63. Found: C, 66.73; H, 6.43; N, 10.55.

4.2.2.7. (2S$_{5}$,1'S)-2-Benzyl-2-[(1'-carbamoyl)ethyl]carbamoyl-1-(2-naphthoxy)sulfonazetidine, (S$_{5}$)-10h

Syrup. Acetone/CH$_2$Cl$_2$, 1:4. Yield: 35%. Mp: 137–139°C. [x]$_D$ = +4.0 (c 0.9, CHCl$_3$). H NMR (DMF-d$_6$): 13.9 (br s, 1H, CONH$_2$), 7.38–7.14 (m, 12H, 1'-NH, Ph, CONH$_2$), 7.06 (br s, 1H, CONH$_2$), 4.26 (m, 1H, 1'-H), 3.63 (m, 1H, 4-H), 3.33 (d, 1H, J = 13.6, 2-CH$_2$), 2.98 (d, 1H, J = 13.8, 2-CH$_2$), 2.98 (m, 1H, 4-H), 2.82 (m, 2H, CH$_2$CH$_2$Ph), 2.34 (m, 2H, CH$_2$CH$_2$Ph), 2.24 (m, 1H, 3'-H), 2.07 (m, 1H, 3'-H), 1.36 (d, 3H, J = 7.4, 1'-CH$_3$). MS: 394.2 [M+1]$^+$. Anal. Calcld for C$_{27}$H$_{27}$N$_2$O$_4$: C, 67.63; H, 6.91; N, 13.72. Found: C, 67.70; H, 7.02; N, 13.67.

4.3. Antiviral assays

The antiviral assays, other than the anti-HIV, -HCMV and -VZV assays, were based on inhibition of virus-induced cytopathicity in HEL (herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus, vesicular stomatitis virus, cytomegalovirus (HCMV))
and varicella-zoster virus (VZV), Vero (parainfluenza-3, reovirus-1, Sindbis and Coxackie B4), HeLa (vesicular stomatitis virus, Coxackie virus B4, and respiratory syncytial virus), CrFK (feline coronavirus (FIPV) and feline herpes virus) or MDCK [influenza A (H1N1; H3N2) and influenza B] cell cultures. Confluent cell cultures (or nearly confluent for MDCK cells) in microtiter 96-well plates were inoculated with 100 CCID50 of virus (1 CCID50 being the virus dose to infect 50% of the cell cultures). After a 1-h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations (fivefold compound dilutions) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Minimal cytotoxic concentration (MCC) of the compounds was defined as the compound concentration that caused a microscopically visible alteration of cell morphology. CC50 values were estimated from graphic plots of the percentage of cytopathogenicity as a function of concentration of the compounds.

4.3.2. Cytostatic toxicity assays

Pathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%. EC50's were calculated from graphic plots of the percentage of cytopathogenicity by 50%.

Cytostatic activity measurements were based on the inhibition of HEL cell growth. HEL cells were seeded at 5 × 10^5 cells/well into 96-well microtiter plates and allowed to proliferate for 24 h. Then, medium containing different concentrations of the test compounds was added. After 3 days of incubation at 37 °C, the cell number was determined with a Coulter Counter. The cytostatic concentration was calculated as the CC50 or compound concentration required to reduce growth by 50% relative to the number of cells in the untreated controls. CC50 values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds. Cytotoxicity was expressed as minimum cytotoxic concentration (MCC) or compound concentration that causes a microscopically detectable alteration of cell morphology.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.052.

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