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Lipophilic peptide inhibitors of ribonucleotide reductase enzyme of herpes simplex virus

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Abstract

Lipophilic modified peptides corresponding to the COOH terminus of subunit 2 of herpes simplex virus ribonucleotide reductase were synthesised and evaluated. Lipidic conjugation on the N-terminus greatly enhanced the peptide inhibitory activity.

Key words: Lipidic amino acid; Lipidic peptide; Herpesvirus ribonucleotide reductase inhibition

1. Introduction

Herpes simplex virus type 1 and 2 ribonucleotide reductase is formed by the association of two non-identical subunits. A peptide corresponding to the C-terminus of subunit 2, Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu, has been shown to inhibit completely the reductase activity. The minimum active core was the Val-Val-Asn-Asp-Leu sequence, however, the activity increased with increasing the peptide chain. The peptides inhibited the activity of the ribonucleotide reductase enzyme of herpes simplex virus in vitro, but unfortunately the peptides did not show inhibitory activity in vivo (Cohen et al., 1986; Gaudreau et al., 1987).

The lipidic amino acids and their homo-oligomers, the lipidic peptides, represent a class of compounds which combine structural features of lipids with those of amino acids (Gibbons et al., 1990). Because of their bifunctional nature, the fatty amino acids and peptides have the capacity to be chemically conjugated to or incorporated into peptides. The resulting conjugates would be expected to possess a high degree of membrane-like character, which may be sufficient to facilitate their passage across membranes (Toth et al., 1994a). The long alkyl side chains may also have the additional effect of protecting a labile parent drug from enzymatic attack (Toth et al., 1994b).

To examine the effect of lipidic conjugation on the biological activity, it was decided to modify the Val-Val-Asn-Asp-Leu peptide sequence with a lipidic amino acid.

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2. Materials and methods

Infrared spectra were recorded with a Perkin Elmer 841 spectrophotometer. ^{1}H-NMR spectra were obtained on Varian XL-300 and Bruker AM500 instruments operating at fields of 300 and 500 MHz, respectively; chemical shifts are reported in ppm downfield from internal TMS. Mass spectra were run on a VG Analytical ZAB-SE instrument, using fast atom bombardment (FAB) ionisation (high resolution FAB-MS accuracy: 1 ppm). Reaction progress was monitored by thin-layer chromatography (TLC) on Keiselgel PF_{254} using dichloromethane/methanol 10:1 and 10:3 as the mobile phase. Purification was achieved by flash chromatography through Kieselgel G (dichloromethane/methanol 10:1). Solvents were evaporated under reduced pressure with a rotary evaporator.

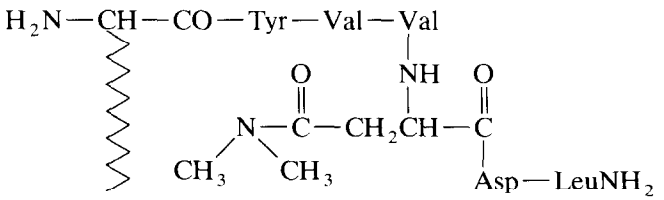
2.1. Peptide purification

Analytical HPLC separation was carried out on a Vydac C_{18} 5 RAC column. Analytical separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing constantly to 60% acetonitrile at 30 min, staying at this concentration for 20 min and decreasing steadily to 0% acetonitrile for 10 min at a constant flow of 0.7 ml min^{-1}. For preparative separation a TSK-gel semipreparative C_{18} column was used. Separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing constantly to 60% acetonitrile at 180 min, staying at this concentration for 60 min and decreasing steadily to 0% acetonitrile for 30 min at a constant flow of 7 ml min^{-1}.

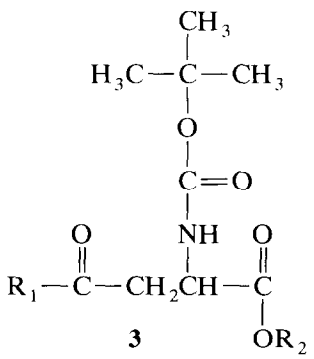
The following compounds were synthesised and used for peptide synthesis: α-benzyl β-2,4,6-trichlorophenyl-*N-tert*-butoxycarbonylaspartinate (**3c**), benzyl *N-tert*-butoxycarbonyl-β-dimethylasparaginate (**3b**), *N-tert*-butoxycarbonyl-β-dimethylasparagine (**3a**), benzyl *N-tert*-butoxycarbonyl-β-(methyl)tetradecanoate-2-yl)asparaginate (**3e**), and *N-tert*-butoxycarbonyl-β-(methyltetradecanoate-2-yl)asparagine (**3d**).

2.2. Synthesis of peptides 1 and 2

The syntheses of peptides were accomplished manually by a stepwise solid-phase procedure on



1



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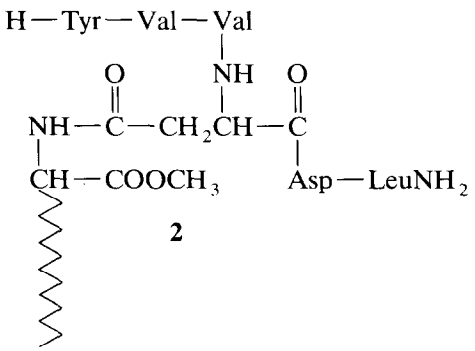

2

3	R_1	R_2
a	$N(CH_3)_2$	H
b	$N(CH_3)_2$	$CH_2C_6H_5$
c	$OC_6H_2Cl_3$	$CH_2C_6H_5$
d	$NHCH[(CH_2)_{11}CH_3]COOCH_3$	H
e	$NHCH[(CH_2)_{11}CH_3]COOCH_3$	$CH_2C_6H_5$

MBHA Novabiochem resins (substitution 0.48 mmol/g resin). The syntheses of the first and every subsequent level of the peptides' construction were achieved using a 4 M excess of preformed symmetrical anhydride of N-Boc amino acids in dichloromethane. The peptides were removed from the resin support using the high HF method. The crude peptides were purified by semipreparative HPLC. The diastereomers were separated by semipreparative HPLC, however, the absolute configurations were not determined and the biological experiments were carried out using the diastereomeric mixtures.

Details of the experimental part and physicochemical data of the compounds (R_f, ^{1}H-NMR, MS) are available on request.

2.3. Enzyme purification

HSV-2 ribonucleotide reductase was partially purified from BHK cells infected with HSV-2 (strain 186) and harvested 7 h post-infection. Cell pellets were resuspended in 5 ml of 20 mM Hepes, pH 7.6, 1 mM DTT and 30% v/v glycerol and then sonicated for 3×20 s with 5 s intervals. The suspension was then centrifuged at 100000 $\times g$ for 20 min. The supernatant was collected and streptomycin sulphate was added to a level of 1% w/v and the mixture stirred for 20 min before being centrifuged at 20000 $\times g$ for 20 min. Ribonucleotide reductase activity was precipitated from the resulting supernatant by the addition of crystalline $(NH_4)_2SO_4$ to 40% saturation (0.242 g/ml). The precipitate was collected by centrifugation as before and the pellet resuspended in the above buffer and desalted using the same buffer and a Sephadex G-25 PD10 column (Pharmacia). The desalted enzyme preparation was stored at $-70°$ until used.

2.4. Ribonucleotide reductase assay

The standard reaction mixture for ribonucleotide reductase in a final volume of 50 μl contained 100 mM Hepes pH 8.1, 100 mM NaF, 10 mM DTT, 0.5 μCi [^{3}H]CDP (10–30 Ci/mmol), 10 μl of enzyme and varying levels of peptide dissolved in 0.2 M Hepes, pH 7.6. Following incubation at 37°C for 1 h the reaction was terminated by heating in a boiling water bath for 15 min. After cooling, the nucleotides were hydrolysed to nucleosides by the addition of 50 μl of bovine intestinal mucosa alkaline phosphatase (Sigma) in 0.1 M glycine, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ (approx. 30 U per assay). The reaction was stopped by adding 150 μl 0.1 M KH_2PO_4 pH 4.7 (containing cytidine and deoxycytidine unlabelled nucleoside markers). The assay mixture was centrifuged at 10000 $\times g$ for 3 min and the supernatant analyzed using an isocratic, reverse-phase HPLC system (LDC Analytical) and a Radiomatics radioactivity flow detector (Canberra Packard). The column was a 10 cm $\times$ 4.6 mm 5 μm Spherisorb ODS2 cartridge with a mobile phase of 0.1 M KH_2PO_4 pH 4.7 running at 1 ml/min and the injection volume was 10 μl. The levels of radiolabelled cytidine and deoxycytidine was measured by integration of the radioactive peaks corresponding to the absorbance readings at 254 nm of the unlabelled markers. Both nucleosides were resolved in 4 min using the above system. IC_{50} values were determined as the concentration of peptide required to give a 50% reduction in enzyme activity compared to control assays.

3. Discussion

Previous studies (Glaxo, unpublished results) showed that certain modifications on the HSV H2 peptide sequences may increase inhibitory activity. The modified peptides, Ac-Tyr-Val-Val-Asn-Asp-Leu-OH and $PhCH_2CH_2CO$-Val-Val-Asn$(CH_3)_2$-Asp-Leu-OH were more active than the H2-(7–15) nonapeptide (Table 2).

Table 1
Partition coefficients (log P) of compounds **1** and **2** (calculated by EluEx version 3.0/C CompuDrug Chemistry, Budapest program)

Compound	Log P	Acid pK_a	Basic pK_a
H2-(7–15)	−8.58	2.9	8.1
2	1.91	2.9	8.1
1	4.85	3.9	8.2

Table 2
Inhibitory potency of HSV analogs

Peptide	IC_{50} (μM)	RI (%)
H2-(7–15)	60	1.00
Peptide **1**	2.3	26
Peptide **2**	> 100	< 0.6
Ac-Tyr-Val-Val-Asn-Asp-Leu-OH	8.5	7.1
$PhCH_2CH_2CO$-Val-Val-Asn-$(CH_3)_2$-Asp-Leu-OH	0.11	545

IC_{50}, concentration of peptide producing 50% of the maximal inhibition; RI, relative inhibitory potency compared to H2-(7–15) = 1.00 (Gaudreau et al., 1987).

We have taken two approaches to modify the lipophilicity of the parent peptide. In the first approach, the hexapeptide H-Tyr-Val-Val-Asn-$(CH_3)_2$-Asp-LeuNH_2 was extended on the N-terminus with 2-aminotetradecanoic acid, resulting in the heptapeptide **1**, (log $P = 4.85$, Table 2), while in the second the peptide was modified on the Asn side chain with 2-aminotetradecanoic acid yielding the heptapeptide **2** (log $P = 1.91$, Table 1).

The peptides **1** and **2** were synthesised according to the solid-phase technique of Merrifield (1963) and their primary structures verified by MS and NMR. The specially protected amino acids were synthesised in the following ways: The synthesis of *N-tert*-butoxycarbonyl-β-dimethylasparagine (**3a**) was accomplished by hydrogenation of benzyl-*N-tert*-butoxycarbonyl-β-dimethylasparaginate (**3b**) which was obtained by reacting active ester **3c** with a dichloromethane solution of dimethylamine at -15°C. **3c** was formed from N-Boc-aspartic acid α-benzyl ester by treatment with 2,4,6-trichlorophenol and dicyclohexylcarbodiimide in dichloromethane. 2-*tert*-Butoxycarbonylaminotetradecanoic acid and methyl 2-aminotetradecanoate were synthesised from 1-bromododecane (Gibbons et al., 1990). *N-tert*-Butoxycarbonyl-β-(methyl)tetradecanoate-2-yl)asparagine (**3d**) was produced by hydrogenation of **3e** which was obtained by reacting N-Boc-aspartic acid α-benzyl ester with methyl 2-aminotetradecanoate and dicyclohexylcarbodiimide in dichloromethane.

The IC_{50} values and the relative inhibitory potencies (RI) are reported in Table 2. The peptide **1** where the N-terminus of the H-Tyr-Val-Val-Asn-$(CH_3)_2$-Asp-Leu-OH sequence was extended with a lipidic amino acid showed moderate inhibitory activity ($IC_{50} = 2.3$ μM), while peptide **2** with a lipidic substitution on the side chain of Asn had very weak activity. Peptide **1** with an octanol/water partition coefficient of 4.85 (Table 2) may be a successful candidate for further in vivo studies.

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