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Design of potent lipophilic-peptide inhibitors of human neutrophil elastase: In vitro and in vivo studies

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Abstract

In keeping with the presence of an extended hydrophobic binding site in human neutrophil elastase (HNE), a series of lipophilic peptide derivatives were synthesized. In vitro studies, using an oligopeptide substrate for HNE, showed that lipidic amino acid derivatives **1b**, **1c** and **1d** were potent HNE inhibitors; the lipophilic amino acid trimer conjugate **1d** ($IC_{50} = 1.8 \times 10^{-10}$) was a more powerful inhibitor than the dimer conjugate **1c** ($IC_{50} = 2.8 \times 10^{-10}$) which was more potent than the monomer conjugate **1b** ($IC_{50} = 2.9 \times 10^{-9}$). When injected intradermally to rabbit dermis, compound **1d** protected skin elastic fibres against degradation by HNE.

Keywords: Human neutrophil elastase; Elastic fiber; Lipophilic peptide

1. Introduction

Human neutrophil elastase (HNE; EC 3.4.21.37) has physiological functions in host defense against bacterial infections and extracellular matrix (ECM) remodelling following tissue injury (Takahashi et al., 1989). During acute inflammatory disorders, its local concentration probably exceeds those of its natural inhibitors such as α_1-proteinase inhibitor, leading to ECM degradation. HNE is implicated in emphysema, adult respiratory distress syndrome, glomerular nephritis, rheumatoid arthritis and its involvement in periodontitis is well documented (Mittman, 1972; Janoff, 1972a, b, 1985; Miyano et al., 1982; Burchardi et al., 1984; Stein et al., 1985; Digenis et al., 1986). HNE inhibitor imbalance can be restored by administrations of natural inhibitor ($\alpha_1 P_i$) or low molecular weight peptidic reversible or irreversible inhibitors (Yasutake and Powers 1981; Kettner and Shenvi, 1984; Hassal et al., 1985; Hori et al., 1985; Groutas et al., 1985; Trainor, 1987).

Peptidic HNE inhibitors have a common hydrophobic peptide sequence, which partially mimics certain amino acid sequences found in elastin. The Ala-Ala-Pro-Val sequence fits the P–P_1 subsites of elastase and inhibits HNE competitively with K_i approx. 10^{-4} M. The covalent coupling of lipophilic moieties as long chain *cis* unsatu-

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rated fatty acids was found to increase considerably the inhibitory capacity of the substance, in keeping with the presence of an unusual hydrophobic binding site in HNE located near its active centre (Hornebeck et al., 1985; Lafuma et al., 1989; Boduier et al., 1991). In order to gain a better insight into the size of this hydrophobic pocket, a series of lipopeptides of increasing lipophilic character were synthesized, keeping the peptide moiety (Ala-Ala-Pro-Val) constant and their HNE inhibitory capacity was then analysed.

These lipidic peptides combine the physicochemical and biological properties of peptides and proteins with those of lipids and membranes and can also confer protection against enzyme degradation to biologically labile compounds (Toth et al., 1994). In that sense, they behave as bifunctional inhibitors.

The investigation also involved the evaluation of the bifunctional inhibitory capacity of one such lipopeptide in vivo, using elastin as the target macromolecule and the resulting data quantified by automated image analysis.

2. Materials and methods

Infrared spectra were recorded with a Perkin Elmer 841 spectrophotometer. Melting points are not given for diastereomeric mixtures. ^{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) techniques. Reaction progress was monitored by thin-layer chromatography (TLC) on Kieselgel PF_{254} using dichloromethane/methanol 10:1 as the mobile phase. Purification was achieved by TLC using Kieselgel $PF_{254+366}$ (Merck) on 20×20 cm plates of thickness 1.5 mm, or column or flash chromatography through Kieselgel G, (system: dichloromethane/methanol 10:1 v/v). Solvents were evaporated under reduced pressure with a rotary evaporator. Infrared spectra gave characteristic NH, carbonyl and aromatic absorbances. Purity of the compounds was determined by TLC and high-pressure liquid chromatography (HPLC). HPLC separation was carried out on a Whatman Partisal 5 RAC silica column. HPLC grade dichloromethane (Aldrich) and methanol (Rathburn) were filtered through a 25 μm membrane filter and degassed with helium flow prior to use. Separation was achieved with a solvent gradient beginning with 0% methanol, increasing constantly to 50% methanol at 15 min and decreasing steadily to 0% methanol from 17 to 20 min at constant flow of 3 ml min^{-1}. The gradient was effected by two microprocessor-controlled Gilson 302 single-piston pumps. Compounds were detected with a Heliochrome UV-Vis detector at 254 nm. Chromatographs were recorded with an LKB 2210 single-channel chart recorder.

2.1. Chemical synthesis

The compounds were synthesised by standard peptide synthesis methods (Bodanszky and Bodanszky, 1984) (experimental details and data are available on request).

2.2. Biological investigations

2.2.1. In vitro studies

HNE (from Elastin Products Co., St Louis, MO) was pure as assessed by SDS-PAGE; it was active site titrated as previously described. The inhibitory capacity of lipopeptides towards HNE amidolytic activity was determined as reported (Hornebeck et al., 1985) using succinoyl-alanyl-alanyl-alanine *p*-nitroanilide as the substrate. Percent inhibition refers to velocity ratios in the presence and absence of inhibitor. IC_{50} is defined as the concentration of lipopeptide required for achieving 50% inhibitions of the enzyme.

2.2.2. In vivo studies

Hair from the back of male white Bouscat rabbit (weighing approx. 1500 g) was first removed and the following solutions were injected intradermally at six different sites:

(1) 200 μl of phosphate-buffered saline (PBS) pH 7.4.

(2) 500 μg of lipopeptide in 1:1 (v/v) EtOH/PBS.
(3) 7.6 μM HNE in 200 μl PBS.
(4) 250 μg of lipopeptide dissolved in 1:1 (v/v) EtOH/PBS followed 15 min later by administration at the same site of 7.6 μM HNE (100 μl solution in PBS).
(5) 7.6 μM HNE (100 μl solution in PBS) followed 15 min later by local administration of 250 μg of lipopeptide dissolved in EtOH/PBS 1:1 (v/v).
(6) Finally, 7.6 μM HNE preincubated for 15 min with 250 μg of lipopeptide and injected intradermally.

Skin biopsies were performed 3 h post-injections. Biopsies were then fixed, dehydrated and embedded in paraffin using routine procedures. Serial sections (5 μm) were performed and elastic fibres were stained using the (+)-catechin dye, a technique which allowed individualisation of the elastic fibre system with colourless background, appropriate for morphometric analysis. Automated image analysis for appreciating the elastolytic potential of a proteinase and/or the inhibitory capacity of an HNE inhibitor has been previously thoroughly described. $A_A\%$ refers to the area fraction occupied by elastic fibres in rabbit skin.

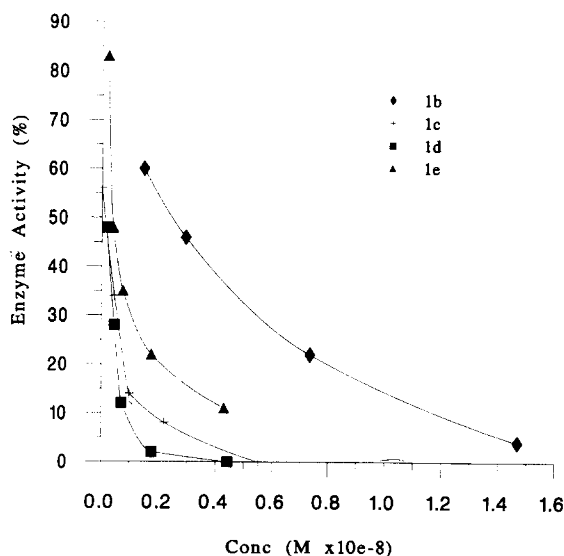

Fig. 1. In vitro HLE inhibitory activity of **1b**, **1c**, **1d** and **1e**.

Table 1
IC_{50} values (giving 50% inhibition of HLE activity) of compounds **1b**, **1c**, **1d** and **1e** (evaluated from the dose-response curves)

Inhibitor	IC_{50} (mmol ml^{-1})
1b	2.9×10^{-9}
1c	2.8×10^{-10}
1d	1.8×10^{-10}
1e	4.1×10^{-10}

3. Results and discussion

Inhibition of HNE amidolytic activity towards $Suc(Ala)_3NA$ by lipopeptides **1b**, **1c**, **1d** and **1e** is shown in Fig. 1 and Table 1. Enzyme inhibition increased with increased lipophilicity of the substances; all compounds were more potent HNE inhibitors than *N*-oleoyl-$(Ala)_2$Pro-Val-OH (1a), for which a literature value was readily available. Compound **1d** inhibited HNE with $IC_{50} = 1.8 \times 10^{-10}$ M as compared with $IC_{50} = 10^{-6}$ M (Hornebeck et al., 1985) for the peptidic moiety above. Such difference indicated, in keeping with the size of the lipidic part of the molecule, that the extended hydrophobic subsites of HNE could accommodate large hydrophobic molecules. Modifying the end carboxylic group of the lipopeptide to an ester (**1e**) led to decreased inhibitory capacity of the compound (Fig. 1 and Table 1). This substantiates earlier studies suggesting a primordial site in ionic interactions between Arg^{217} in HNE and the free carboxylate of the enzyme.

X-L-Ala-L-Ala-L-Pro-L-Val-Y

1

1	**X**	**Y**
a	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO$	OH
b	$(CH_3)_3COCO$-DL-$NH[CH(CH_2)_{11}CH_3]$	OH
c	$(CH_3)_3COCO\{$DL-$NH[CH(CH_2)_{11}CH_3]CO\}_2$	OH
d	$(CH_3)_3COCO\{$DL-$NH[CH(CH_2)_{11}CH_3]CO\}_3$	OH
e	$(CH_3)_3COCO\{$DL-$NH[CH(CH_2)_{11}CH_3]CO\}_3$	OCH_3

Scheme 1.

Fig. 2. Control: 0.2 ml PBS pH 7.4.

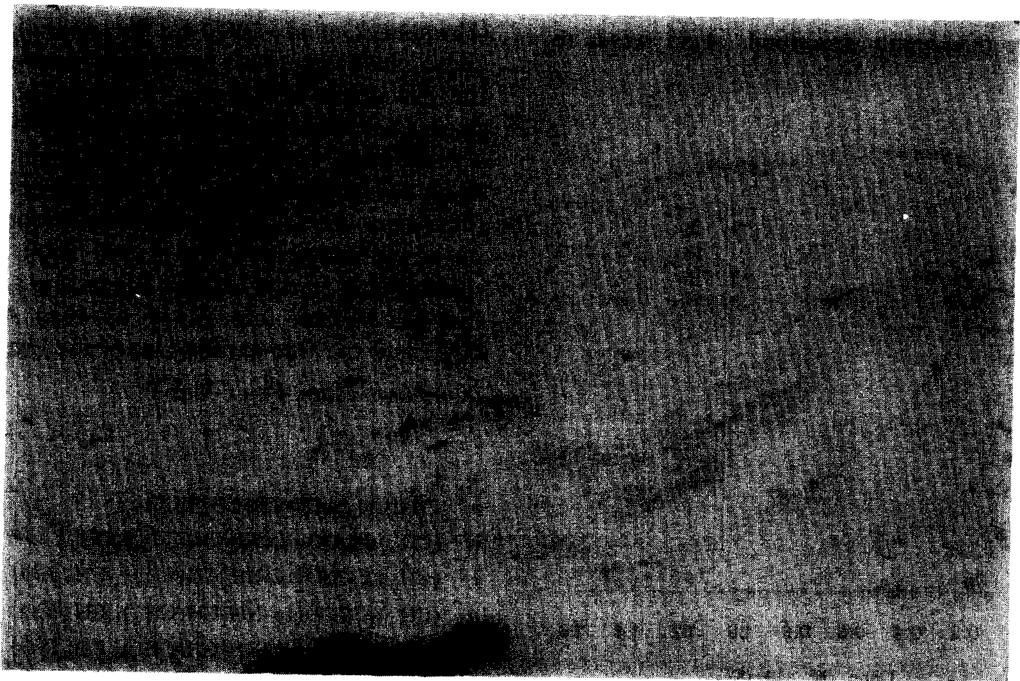

Fig. 3. HLE: 7.6 μM HLE in 0.2 ml PBS.

Table 2
In vivo enzyme inhibitory activity of compound 1d

	A_0 average %	S.D.
(1) PBS	5.44	+0.9
(2) **1d**	6.62	+1.3
(3) HLE	2.20	+0.9
(4) **1d** 15 min + HLE	5.95	+2.7
(5) HLE 15 min + **1d**	7.05	+1.3
(6) HLE + **1d** (simultaneously)	7.76	+1.3

The in vivo HNE inhibitory capacity of 1d as well as its protective functions against elastolysis by elastin was further investigated (Table 2, $p > 0.0001$). Intradermal injections of lipopeptide 1d did not modify $A_A\%$ of rabbit skin elastic fibres, as compared to animals where only PBS was administered (Fig. 2). HNE administrations, under similar conditions, resulted in a significant reduction in dermal elastic fibre levels (Fig. 4). When 1d was administered prior (15 min), with or after HNE, it could protect elastic fibre degradation induced by the enzyme (Fig. 3). Such results extend previous studies demonstrating the in vitro, ex vivo and in vivo bi-functionality of lipopeptides acting as potent elastase inhibitors and elastic fibre protectors against elastolysis.

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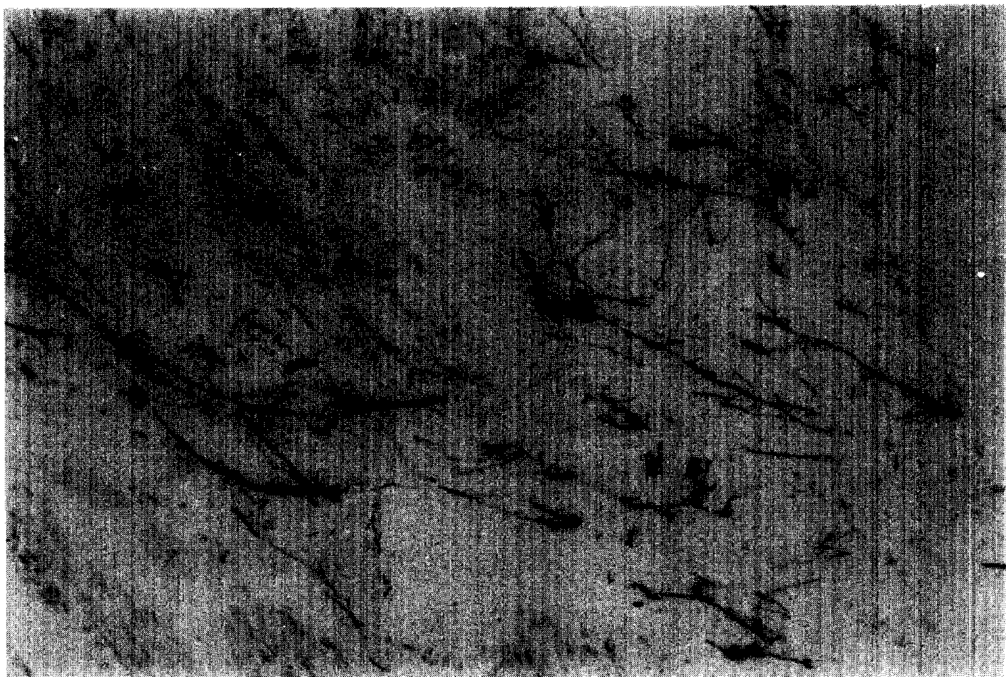

Fig. 4. HLE + inhibitor **1d**: 7.6 μM HLE, 250 μg **1d** in 50 μl PBS/EtOH 1 : 1.

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