Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 993–998

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE DESIGN AND SYNTHESIS OF INHIBITORS OF THE CYSTEINYL PROTEASE, *DER P* I.

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Received 25 November 1997; accepted 19 March 1998

Abstract: Prototype irreversible inhibitors of the cysteinyl protease *Der p* I were designed, synthesised and evaluated *in vitro*. Candidates were designed using a modular approach, whereby a peptide sequence was appended with known thiophilic moieties. This hinged on utilizing peptide sequences from substrate specificity data compiled using proprietary RAPiD™ technology.

Introduction

Faecal extracts from the house dust mite (*Dermatophagoides pteronyssinus*[1]) are reported to give rise to extrinsic, allergic rhinitis and atopic dermatitis in humans,[2,3] and are associated with the development of juvenile asthma.[4] They have been shown to elicit an IgE antibody response in 80% of patients suffering from house dust mite allergy.[5,6] It has recently been shown that the proteolytic activity of the enzymes present in the extracts from *D. pteronyssinus* appears to be closely associated with allergic disease in susceptible individuals.[7]

The cysteinyl protease *Der p* I has recently been isolated from the faecal pellets of *D. pteronyssinus*.[8] *Der p* I has been shown to cleave the low affinity CD23 receptor for IgE selectively from the surface of human cultured B-cells (RPMI 8866 B cell line)[9] and EBV-transformed human B cell line BC5.[10] This cleavage occurs at two sites, ^{298}Glu-^{299}Ser and ^{155}Ser-^{156}Ser.[12] Intact CD23 on B lymphocytes modulates local IgE synthesis,[12-15] delivering a negative IgE regulatory response to the lymphocyte when the receptor is occupied with IgE.[13,16] *In vitro* it appears that the soluble CD23 produced from the cleavage reaction also upregulates IgE synthesis.[17] Thus it is believed that the allergic response is exacerbated by the proteolytic cleavage mediated by *Der p* I. Laing and Shakib suggested that this could result in potentiation of the IgE mediated response to other allergens.[9] This therefore implied that a designed, selective inhibitor of this enzyme could be used as a potential therapeutic agent to alleviate the allergic response to house dust mites[19] Interestingly, it has also been shown that *Der p* I cleaves the serpin, α_1-antitrypsin between ^{12}Asp-^{13}Thr as well as ^{6}Asp-^{7}Ala.[18] α_1-Antitrypsin protects the lower respiratory tract against proteases released in the lung during inflammation.

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0960-894X/98/$19.00
PII: S0960-894X(98)00151-6

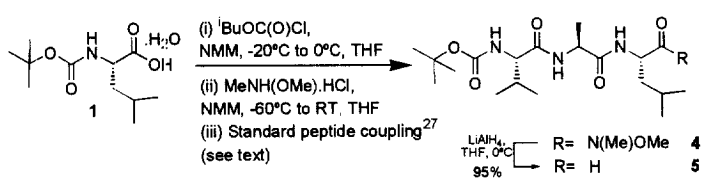
The design of prototype, irreversible inhibitors of this cysteinyl protease was based on a modular, structure-based drug design approach. Initial mapping of the enzyme's substrate specificity identified potential targetting motifs. A chemoselective functionality, expected to form a covalent bond with the active site cysteine, was then attached to the *C*-terminal end of this sequence in order to furnish the inhibitor candidate.

The targeting sequence was identified using RAPiD™ a proprietary technology that utilises combinatorial chemistry to define the substrate specificity of proteolytic enzymes.[20] This approach hinges on the construction of a spatially addressable, quenched fluorescence substrate library using solid phase synthesis techniques and subsequent rapid screening of library components upon release in solution. Thus *Der p* 1 was shown to have high catalytic activity and specificity for several different substrates. For instance, the substrate containing the sequence Val-Ala-Leu-Ser, (k_{cat}/K_M= 3.4 × 10^4 $M^{-1}s^{-1}$, K_M= 7.0μM), cleaved between Leu and Ser as was evident from hplc/ms data of cleavage products.

Functional groups that act as Michael acceptors have previously been utilised as thiophilic agents for the selective inhibition of cysteinyl proteases over other proteolytic classes.[21,22] In addition, it was envisaged that vinylogous esters and sulfones could be readily prepared by elaboration of the aldehyde derived from the peptide targeting motif (itself a potential inhibitor). Acyloxy methyl ketones are another class of thiophilic moieties that have successfully been incorporated into selective inhibitors for this type of enzyme.[23] Peptides appended with this grouping can also be readily made.

Chemistry

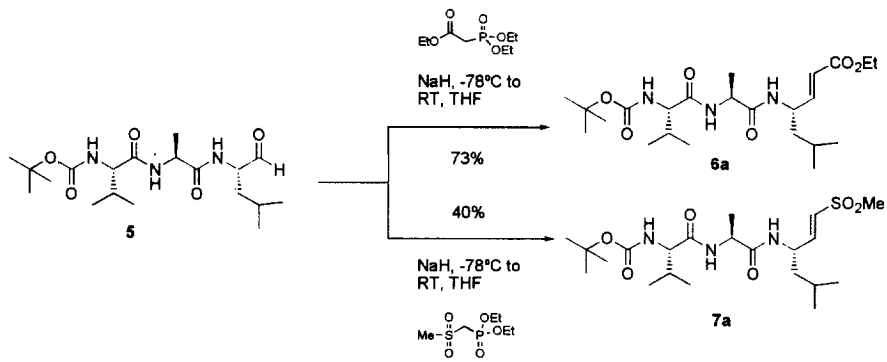
The *E*-vinyl sulphones of the type **7a** and the *E*-vinylogous ethyl ester **6a** were prepared by the Horner-Emmons reaction of intermediate aldehyde **5** with appropriate phosphonate anions (scheme **2**).[24] The aldehyde **5** was obtained by LAH reduction[25] of the corresponding Weinreb amide, **4**.[26] This was prepared in several steps as shown in scheme **1**: Coupling BOC-Leu-OH **1** with *N,O*-dimethylhydroxylamine using isobutylchloroformate activation furnished the Weinreb amide **2**. The BOC group was then removed under acidic conditions.



Scheme 1

Neutralisation of the resulting salt and subsequent coupling to BOC-Ala-OH, *via* formation of the isobutyl mixed anhydride, proceeded smoothly. The resulting dipeptide **3** was deprotected as described above and the free amine

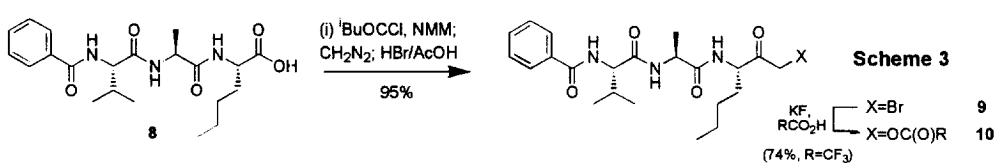
reacted with BOC-Val-OSuc in order to form the tripeptide Weinreb amide **4**. The peptide synthesis proceeded cleanly and only the final Weinreb amide **4** and aldehyde **5** were purified using flash chromatography on silica. No epimer of the aldehyde was evident from the ^{1}H NMR spectrum of the isolated product.



Scheme 2

Sequences with differing *N* terminal capping groups were prepared by removal of the BOC group from the ester under acidic conditions (as above) followed by acylation with the appropriate anhydride.

Peptides appended with the acyloxy methyl ketone grouping were based on the sequence Val-Ala-Nle. A computational model of the enzyme[28] suggested that Nle might better fill the hydrophopic S1 pocket than Leu. However, the quenched fluorescence substrate containing the sequence Val-Ala-Nle-Ser has k_{cat}/K_M 3.5 X 10^4 $M^{-1}s^{-1}$, K_M= 12μM and so exhibits similar kinetics to the Leu analogue. Bz-Val-Ala-Nle-OH (**8**) was prepared using standard solid phase peptide synthesis techniques.[29] This was purified by HPLC before conversion to the bromomethyl ketone (**9**) by treatment of the mixed anhydride with diazomethane followed by HBr in AcOH. The acyloxy methyl ketones were then generated by treatment of the bromide with the appropriate carboxylic acid in a KF mediated coupling, Scheme **3**.



Scheme 3

Enzymology

Purification of Der *p I*

Der p I was purified from either *Dermatophagoides pteronyssinus* faecal mite extract (Smith-Kline Beecham Pharmaceuticals, Harlow, Essex) or from spent mite culture (Dr. T. Merret, Allergy Analysis Centre, Diagnostics

Products Co. Ltd., Llanberis, Gwynedd) using 4C1 antibody (Indoor Biotechnology, Deeside, Clwyd) affinity purification using the method of Hewitt.[10]

Der p I assays were routinely carried out at 25°C in 50 mM potassium phosphate; pH 8.25 containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) with the quenched fluorogenic substrates that contain the *3*-nitrotyrosine/*2*-aminobenzoyl fluorescence/quencher system. Product formation was monitored with respect to time by measuring the increase in fluorescence emission at 420 nm and exciting at 320nm (slits set at 5 nm; PMT set at 700 V). Stock solutions of the various substrates and/or inhibitors were made up in 100% dimethyl sulfoxide and diluted directly into assay buffer prior to use.

Inhibition kinetics for Der p I

Inhibition kinetics were carried out according to previously described methods.[23,30,31] Generally 1 mL of assay buffer containing 12.5 μM substrate containing between 50-1000 nM inhibitor was incubated at 25°C prior to initiation of the reaction by addition of 10 nM *Der p* I. In this case the sensitivity of the fluorimeter was increased by altering the slit widths and photomultiplier voltage (5 nm excitation; 10 nm emission; 950 V).

$$[\text{Product}] = v_s t + (v_o - v_s)[1-\exp(-k_{app}\, t)]/\, k_{app} + d \qquad \textbf{Eq. 1}$$

$$\text{second order rate constant} = (k_{app}/[I])(1+[S]/K_M) \qquad \textbf{Eq. 2}$$

The apparent inactivation rate constant (k_{app}) was calculated by computational least square regression analysis using **Eq. 1**; where v_o is the initial velocity of the reaction, v_s is asymptotic steady-state velocity of the reaction, d is the intercept at time zero. The second order rate was calculated using **Eq. 2**.[23a]

Discussion

Table **1** lists prepared compounds that were potent inhibitors of *Der p* I. E-64 ([*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-amido(4-guanido)butane), a class specific inhibitor of cysteinyl proteases was also tested ($k_{obs}/[I] = 2.25 \times 10^3\ M^{-1}S^{-1}$).[32] The intermediate aldehyde **5** was a potent inhibitor of the enzyme (k_i=14nM). The bromo compound, **9**, also exhibited reversible inhibition kinetics (K_i=1.6nM.)

It has been shown that solely optimising targeting sequences does not always lead to potent and selective inhibitors of cysteinyl proteases.[33] Peptidyl vinyl sulphones have recently been shown to be potent and selective inhibitors of cysteinyl proteases, especially cathepsins O2, S and L, optimal inhibitors having $k_{obs}/[I]$ values in excess of $10^7\ M^{-1}s^{-1}$ [21] It was particularly disappointing to note the relatively poor inhibition by this class of compound in the present study. This was in contrast to the efficacy of the acyloxy methyl ketone series of

inhibitors, being significantly more potent than the vinyl sulphone and vinyl ester series (it seems unlikely that this is due primarily to the substitution of Leu for Nle).Additionally, it was found that these inhibitors were selective for *Der p* I over other cysteinyl proteases. Compound **10b**, for instance, has relative k_{obs}/[I] =2490 and 750 for papain and cathepsin B compared to *Der p*I.

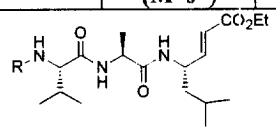
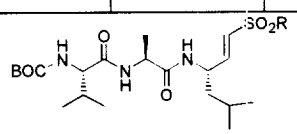
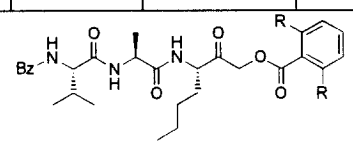
Entry		k_{obs}/[I] ($M^{-1}s^{-1}$)	Method
			
	R		
6a	BOC	1.7×10^3 7.9×10^3	A A
6b	H	$<10^2$	A
6c	Ac	$<10^2$	A
6d	Bz	4.1×10^3 1.6×10^4	A A
			
	R		
7a	Me	1.1×10^3 1.1×10^4	A A
7b	Bn	2.3×10^3 3.5×10^5	A A
7c	Ph	6.4×10^3 2.5×10^4	A A
			
	R		
10a	H	3.7×10^5 5.0×10^4	A A
10b	CH_3	1.6×10^7	B
10c	Cl	6.8×10^7	B
10d	CF_3	1.3×10^7 1.0×10^6 2.2×10^6	B A A

Table 1 *Abbreviations: BOC=t-butoxycarbonyl, Ac=acetyl, Bz=benzoyl*
Values in the table were determined by method A (ref 23, generally n=2, values shown) and/or method B (ref 31 generally n=1). K_{obs} was found to be proportional to [I]

In summary, this *Letter* details the first designed inhibitors of *Der p* I. It illustrates that a modular approach which combines peptide binding sequences, elucidated from substrate libraries, with warheads, designed to irreversibly and selectively react with the active site nucleophile, is an effective strategy for the rapid generation of potent inhibitors for this enzyme.

Acknowledgements. We would like to thank Dr. F. Shakib (University of Nottingham) and Dr. P. Laing (Peptide Therapeutics) for helpful discussions.

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