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Comparison of the binding specificity of two bacterial metalloproteases, LasB of *Pseudomonas aeruginosa* and ZapA of *Proteus mirabilis*, using *N*-alpha mercaptoamide template-based inhibitor analogues

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ABSTRACT

The metalloproteases ZapA of *Proteus mirabilis* and LasB of *Pseudomonas aeruginosa* are known to be virulence factors their respective opportunistic bacterial pathogens, and are members of the structurally related serralysin and thermolysin families of bacterial metalloproteases respectively. Secreted at the site of infection, these proteases play a key role in the infection process, contributing to tissue destruction and processing of components of the host immune system. Inhibition of these virulence factors may therefore represent an antimicrobial strategy, attenuating the virulence of the infecting pathogen. Previously we have screened a library of *N*-alpha mercaptoamide dipeptide inhibitors against both ZapA and LasB, with the aim of mapping the S1′ binding site of the enzymes, revealing both striking similarities and important differences in their binding preferences. Here we report the design, synthesis, and screening of several inhibitor analogues, based on two parent inhibitors from the original library. The results have allowed for further characterization of the ZapA and LasB active site binding pockets, and have highlighted the possibility for development of broad-spectrum bacterial protease inhibitors, effective against enzymes of the thermolysin and serralysin metalloprotease families.

1. Introduction

Extracellular secreted metalloproteases are widely distributed amongst bacteria, and have been identified in both Gram-positive and Gram-negative species. The proposed function of such proteolytic enzymes in bacterial physiology has been postulated as the digestion of complex protein substrates in the surrounding environment, to provide simple nutrients for the bacterial cell [1]. However, many extracellular metalloproteases from pathogenic organisms that have been investigated to date have revealed them as important contributors to virulence [2].

Two major families of bacterial metalloproteases have been identified, the thermolysin (M4) and serralysin (M10) families. Of all the bacterial metalloproteases the most thoroughly characterized is thermolysin, a heat stable metalloprotease produced by *Bacillus thermoproteolyticus* [2]. Although structurally related to the thermolysin family, serralysins differ in that they exploit three histidine residues and a tyrosine to coordinate the zinc ion in the active site, whereas thermolysin-like metalloproteases use two histidine and a glutamic acid residue [3]. Other differences in the active site also exist, for example in serralysins the zinc binding motif extends to HEXXHXXGXXH, whereas thermolysins have a consensus sequence NEXXSD [4,5]. Serralysin metalloproteases are generally inhibited by conventional metal-chelating agents, such as EDTA and thiols, but they are insensitive to phosphoramidon and Zincov, which are specific inhibitors of the thermolysins [6].

Two specific examples of bacterial metalloprotease virulence factors are LasB (also known as Pseudolysin or Pseudomonas elastase) produced by *Pseudomonas aeruginosa*, and ZapA (also known as Mirabilysin) produced by *Proteus mirabilis*. These metalloproteases are members of the thermolysin and serralysin families respectively. It has been demonstrated that each of these bacterial proteases functions as a virulence factor during the process of infection, each having a deleterious effect on the host including the degradation of a broad range of host tissue proteins, and biomolecules involved in innate immunity such as immunoglobulins, complement factors, antimicrobial peptides and cytokines [7–12]. In addition, LasB acts within the bacterial cell as a regulator of polysaccharide (alginate) secretion [13]. This extracellular polysaccharide (EPS) is a key constituent of the pseudomonal biofilm [14], a protected mode of growth where antimicrobial tolerance can be up to 1000-fold that of a planktonic population [15].

P. aeruginosa is recognized as a key pathogen in chronic infection of the cystic fibrosis (CF) lung with such infections displaying

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a characteristic "vicious-cycle" of inflammation and tissue destruction, impeding the normal clearance of bacteria. LasB is a potential underlying trigger of this sustained inflammatory environment due to its ability to cause direct damage of host tissue and processing of immune system components, and also its important role in biofilm formation, leading to an infection highly resistant to antibiotic therapy [13,16–20].

We have recently have characterized the importance of ZapA as a virulence factor in a rat model of *P. mirabilis* induced acute and chronic prostatitis, and have found this virulence factor essential in progression to chronic infection [21]. The underlying mechanism of virulence attributed to the production of ZapA was not found to be biofilm mediated, unlike role of LasB in *P. aeruginosa* biofilms [13,21].

Due to the contribution of these two important metalloproteases to the virulence of their respective bacterial pathogens, inhibition of ZapA and LasB could represent novel anti-virulence targets for therapeutic intervention. The inhibition of bacterial virulence factors would present an antimicrobial strategy that is non-destructive, attenuating virulence mechanisms without directly challenging bacterial cell viability. Such an approach would potentially prevent the emergence of resistant strains, as little or no selective pressure is placed on the bacterial cell.

We have previously reported, the synthesis and kinetic characterization of a library of *N*-alpha mercaptoamide dipeptides as inhibitors of both ZapA and LasB, and this has revealed important information regarding the binding preferences of these enzymes, and has highlighted both striking similarities and important differences between the two. Both metalloproteases exhibited a preference for large aromatic residues in the P1′ position of the dipeptide inhibitor, and aliphatic residues in P2′. However, for ZapA, the ability of the aromatic residue in P1′ to hydrogen bond appeared important in inhibitor binding, whereas this was not important for the inhibition of LasB [22–24].

It is proposed that derivitizing these inhibitors with functionalities lending a high degree of non-polar character, such as halogens, binding to the active site of LasB and ZapA might be improved. In addition, derivatization with halogens may also improve inhibitor binding to ZapA through the process of halogen bonding, an interaction that is analogous to hydrogen bonding [25,26]. This study investigates several analogues of inhibitors from the original *N*-alpha mercaptoamide dipeptide library. These analogues contained halogenated or otherwise substituted phenylalanine to assess the effect of substitution of the aromatic ring on inhibitor performance. Cyclohexyl-L-alanine (CHA) was also used to assess the effects of the loss aromaticity from the inhibitor structure.

2. Materials and methods

2.1. Materials

3,4-Dimethoxy phenylalanine (DMOPA) was obtained from Sigma–Aldrich (Dorset, UK). All remaining amino acids, amino acid analogues, *S*-trityl-mercaptoacetic acid, and the coupling reagents *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt), were obtained from Peptides International (Louisville, USA). Rink amide resin (200–400 mesh) was obtained from Bachem (UK) Ltd. (Meyerside, UK). Dimethylformamide (DMF), dichloromethane (DCM), piperidine, and diisopropylethylamine (DIPEA) were obtained from Sigma–Aldrich (Dorset, UK).

LasB (Pseudolysin) was obtained from Elastin Products Company (MO) and stored at −80 °C in aliquots of 100 μg/ml stock solution in assay buffer containing 0.05 M Tris–HCl, 2.5 mM $CaCl_2$, pH 7.2, until required. ZapA was purified directly from culture supernatants of *P. mirabilis* BB2000, as described previously [23], and stored in at −80 °C in aliquots of 100 μg/ml stock solution in assay buffer containing 0.05 M Tris–HCl, 2.5 mM $CaCl_2$, pH 8.0.

The fluorogenic substrate, Aminobenzoyl-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide, was supplied by Peptides International (Louisville, USA).

2.2. Synthesis of inhibitors

Inhibitors were synthesized using a methodology previously described [22,27], using a CEM Liberty microwave assisted peptide synthesizer and standard Fmoc solid-phase peptide synthesis protocols on Rink amide resin. Amino acid analogues were incorporated under standard coupling conditions. The identity and purity of all the synthesized inhibitors were confirmed by electrospray mass spectrometry and reverse phase (C18) HPLC analysis. Structures of synthesized inhibitor derivatives are illustrated in Fig. 1.

2.3. Kinetic screening

Each inhibitor was screened against both LasB and ZapA using a microtitre-based fluorimetric assay. Hydrolysis of the fluorogenic substrate Aminobenzoyl-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide by each metalloprotease was carried out in assay buffer (50 mM Tris–HCl, 2.5 mM $CaCl_2$, pH 7.2 for LasB; 50 mM Tris–HCl, 2.5 mM $CaCl_2$, pH 8.0 for ZapA). Changes in fluorescence were monitored by a BMG FLUOstar OPTIMA fluorescence microtitre plate reader at an excitation wavelength of 330 ± 10 nm and an emission wavelength of 460 ± 10 nm. Each inhibitor was investigated over a concentration range between 1 μM and 50 μM in order to determine accurate kinetic parameters (K_i). Previous observations have revealed that these inhibitors do not fit the standard linear transformation required for determination of K_i via Michaelis–Menton kinetics [22,23], therefore K_i values were

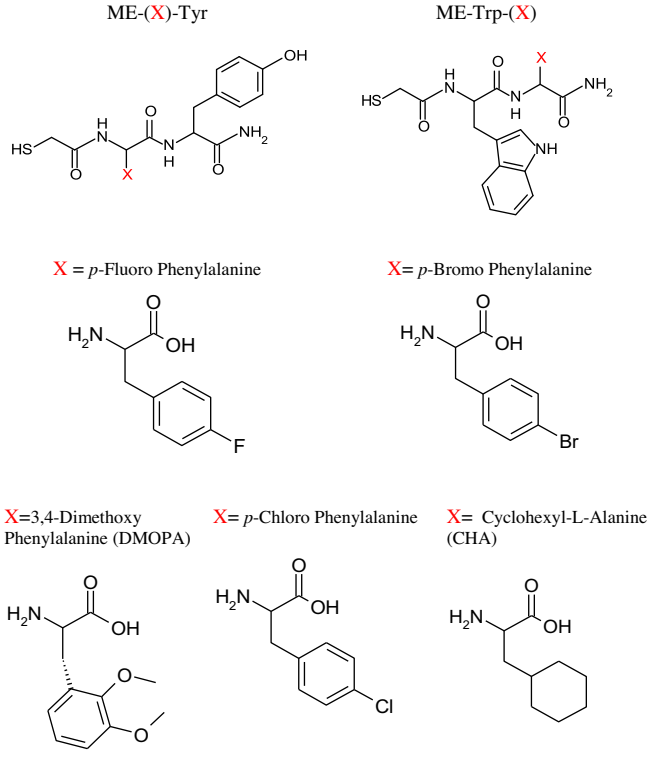

Fig. 1. Structures of synthesized *N*-alpha mercaptoamide inhibitor analogues.

calculated using the Morrison equation for tight binding inhibitors, on GraphPad Prism software (version 5). An example of a typical progress curve for the inhibition of protease-mediated substrate hydrolysis is shown in Fig. 2.

3. Results

3.1. Inhibitor analogue screening against ZapA

During the screening of the *N*-alpha mercaptoamide dipeptide inhibitors against LasB by Cathcart et al. [22,24], it was noted that lead compounds possessed non-polar side chains that were often bulky and aromatic. It was proposed that derivatization of these compounds with functionalities lending a high degree of non-polar character, such as halogens, binding to the active site of LasB might be improved. Fluorinated functionalities are becoming more common in drug candidates, due to the strong electron-withdrawing effects of the halogen atom, which can produce favourable effects on interaction with target proteins. Examples of organofluorine compounds currently in clinical use include Lipitor® (atorvastatin), Prozac® (fluoxetine) and Ciprobay® (ciprofloxacin) [28]. Considering the similarities between lead compounds identified for ZapA and LasB, as discussed previously, these inhibitor analogues were also screened against ZapA. These analogues included those with substituted phenylalanine residues, or the non-aromatic cyclohexyl-L-alanine.

The results presented in Table 1 demonstrate that for the inhibition of ZapA, substitution of phenylalanine with chlorine or bromine has a beneficial influence on K_i in the P1′ position, when compared to the parent compound, SH–CH_2–CO–Phe-Tyr–NH_2. DMOPA or *p*-fluoro phenylalanine showed no improvement, while loss of aromaticity with CHA in P1′ has a largely detrimental effect on K_i, indicating that aromatic side chains are of particular importance at the S1′ binding pocket of ZapA. The improvement in K_i as shown by SH–CH_2–CO–Phe(Cl)–Tyr–NH_2 and SH–CH_2–CO–Phe(Br)–Tyr–NH_2 is possibly due to the electron-withdrawing effect of halogen substituents in the aromatic ring. These atoms are strongly electronegative, attracting the electron cloud from the aromatic ring, leaving a partial positive charge, which can in turn participate in electrostatic interactions through the attraction of lone electron pairs on another heteroatom. Alternatively interaction with the active site of ZapA may also be a result of the halogen substituent participating in halogen bonding. Halogen bonding (XB) is a direct parallel to hydrogen bonding (HB), the difference being that in HB a hydrogen acts as an electron acceptor, while in XB the halogen acts as an electron acceptor. Chlorine, bromine and iodine all participate in halogen bonding, although it is not thought that fluorine can form halogen bonds, but are more likely to serve as hydrogen bond acceptors [25,26]. This may explain the failure of *p*-fluoro phenylalanine in P1′ to improve K_i in the same manner as *p*-bromo phenylalanine or *p*-chloro phenylalanine.

Table 1
K_i values (μM) for the *N*-alpha mercaptoamide parent inhibitors and inhibitor analogues against ZapA of *Proteus mirabilis* and LasB of *Pseudomonas aeruginosa*.

Parent inhibitor	ZapA K_i (μM)	LasB	Inhibitor analogue	ZapA K_i (μM)	LasB
ME-Phe-Tyr	10.50	0.04	ME-Phe(Cl)-Tyr	6.15	0.31
			ME-Phe(Br)-Tyr	7.73	0.03
			ME-DMOPA-Tyr	13.38	1.04
			ME-Phe(F)-Tyr	17.67	0.04
			ME-CHA-Tyr	56.53	0.51
ME-Trp-Phe	38.52	11.00	ME-Trp-CHA	5.87	0.16
			ME-Trp-DMOPA	7.86	4.30
			ME-Trp-Phe(Cl)	8.52	0.02
			ME-Trp-Phe(Br)	8.91	0.03
			ME-Trp-Phe(F)	17.84	0.05

All inhibitor analogues with the derivatized phenylalanine or CHA in the P2′ position improved the K_i over that of the parent compound, SH–CH_2–CO–Trp-Phe–NH_2. The mechanism by which these inhibitor analogues improve inhibitor binding is much more difficult to assess than those previously discussed with modifications in the P1′ position. This is due to the range of both aliphatic and aromatic residues found in the P2′ position of the inhibitors that performed well against ZapA, as reported in our previous investigation [23]. However, it is clear that by modification of the parent compound with these substituted phenylalanine residues or CHA has improved binding to the S2′ pocket of the ZapA active site, and is possibly a result of non-specific hydrophobic interaction.

3.2. Inhibitor analogue screening against LasB

Table 1 also reveals that in the case of LasB, halogenated inhibitor side chains had a positive effect in inhibitor binding when used in the P2′ position, in agreement with the preference for bulky

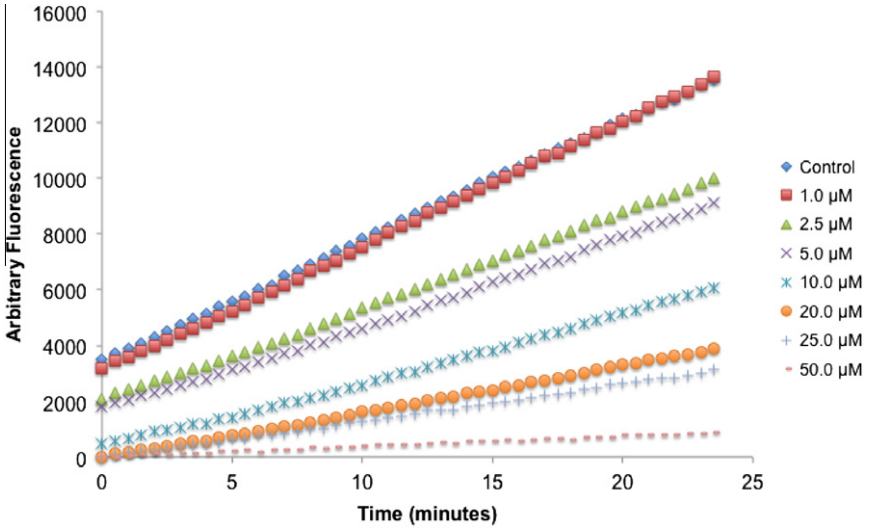

Fig. 2. Typical progress curves for hydrolysis of Aminobenzoyl-Ala-Gly-Leu-Ala-*p*-Nitrobenzyl-Amide by ZapA in the presence of a range of concentrations of SH–CH_2–CO–Phe(Cl)-Tyr–NH_2.

hydrophobic groups in the S2′ binding pocket of LasB reported previously [22]. In this position, the halogenated phenylalanine surpasses the parent compound SH–CH_2–CO–Trp-Phe–NH_2 (K_i = 11.00 μM) with K_i values in the nano molar range (SH–CH_2–CO–Trp-Phe(Cl)–NH_2 K_i = 0.02 μM, SH–CH_2–CO–Trp-Phe(Br)–NH_2 K_i = 0.03 μM, and SH–CH_2–CO–Trp-Phe(F)–NH_2 K_i = 0.05 μM), possibly a result of the electronegative properties of the halogen substituent, as discussed above. Halogenated residues in the P1′ position pose little benefit in inhibitor binding to LasB, in fact *p*-chloro phenylalanine in P1′ imposes a detrimental effect on inhibitory potency, with a K_i of 0.31 μM in comparison to the parent compound SH–CH_2–CO–Phe-Tyr–NH_2, K_i = 0.04 μM.

The K_i values obtained for the non-aromatic CHA in P1′ suggest that aromaticity is important for optimal inhibitor binding. DMOPA also has a deleterious effect on binding to the LasB S1′ pocket, likely due to the introduction of a degree of polarity to the P1′ position of the inhibitor, and we have previously documented a preference for non-polar hydrophobic residues in the LasB S′1 binding pocket [22,24].

4. Discussion

In this study we have expanded on our previous knowledge of the binding preferences of both ZapA and LasB, two distinct bacterial metalloprotease virulence factors from the serralysin and thermolysin families respectively. Although members of different metalloprotease families, these two proteases are structurally related, both belonging to protease clan MA. These fundamental differences, yet underlying similarities, have been reflected in our previously reported data [22–24], and further supported by this study by direct comparison of ZapA and LasB using a number of inhibitor analogues, developed from our original library of *N*-alpha mercaptoamide dipeptides.

For both ZapA and LasB, the inhibitor P1′ residue has been confirmed as the most important structural feature of the inhibitor dictating binding specificity [22–24], however inhibitor screening studies have highlighted a subtle, yet important difference between these two proteases. While inhibitors containing aromatic residues in P1′ have proved superior for both, ZapA appears to require an aromatic residue with the ability to hydrogen bond [23]. This present study has confirmed our previous observations using halogenated phenylalanine residues in P1′. These halogenated residues convey the ability to halogen bond (analogous to hydrogen bonding) with the S1′ pocket of the ZapA active site, dramatically improving the K_i over that of the parent compound containing unsubstituted phenylalanine. With LasB, the effect of introducing a halogenated residue at P1′ did not produce as dramatic an effect, although replacing the aromatic amino acid with CHA had a negative effect on binding. The negative effect of CHA in P1′ was also seen with ZapA.

While we have previously concluded that the P2′ residue is not a driving determinant of inhibitor binding in either LasB or ZapA, this study has demonstrated that with a favourable residue in P1′, binding can be improved through the introduction of a halogenated aromatic residues in P2′; a trend observed for both proteases.

The trends highlighted by this study, taken in consideration with previously published observations [22–24], indicate that it may be possible to design a single active-site directed inhibitor that may be effective against metalloprotease virulence factors of different protease families, and from different species of bacteria. The implications of such a development would be broad-spectrum, anti-infective strategy, the mechanism of which is an anti-virulence approach, rather than causing bacterial death or damage, therefore the development of resistance to such therapeutic strategies is considered less likely to develop than with conventional antibiotics. Given that many extracellular secreted bacterial metalloproteases acting as virulence factors during infection share common evolutionary origins [29], it is anticipated that an extended range of such virulence factors from a variety of bacterial pathogens may be targeted using the fundamental principles highlighted in this study.

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