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Identifying common metalloprotease inhibitors by protein fold types using Fourier Transform Mass Spectrometry

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Abstract—Fourteen natural products, known to inhibit other proteins of the Zincin-like fold class, were screened for inhibition of the Zincin-like fold metalloprotease thermolysin using mass spectrometry. Fourier Transform Mass Spectrometry was successful in identifying actinonin, a known inhibitor of astacin and stromelysin, to be an inhibitor of thermolysin. Molecular modelling studies have shown that specificity within the Zincin-like fold is determined by Protein Fold Topology.

Classification of proteins by different properties including function, fold and sequence has provided a tool to guide efforts towards drug discovery. A correlation between the biosynthetic enzyme synthesising a particular natural product and the therapeutic target of the compound has recently been discovered. Three biosynthetic enzymes in the flavonoid biosynthetic pathway and protein kinases were found to share a similar topological arrangement of different secondary structures around the active site, a phenomenon called Protein Fold Topology (PFT).[1] The biosynthetic enzymes and target had different fold classifications according to the Structural Classification of Proteins (SCOP) database.[2] PFT is a fundamental descriptor unrelated to sequence, sequence family or fold. An examination of natural product inhibitors of Zincin-like fold proteins has demonstrated the conservation of this correlation between different fold targets of the same natural product.[3]

The investigation of natural product inhibitors of Zincin-like fold proteins highlighted specificity within the fold type.[3] To investigate the reason for specificity within the Zincin-like fold, we focused on thermolysin, selected as a typical Zincin-like fold protein. A selection of 14 known natural product inhibitors of the Zincin-like fold metalloproteases (Table 1)[4,5] were tested as inhibitors of thermolysin using affinity mass spectrometry techniques to observe protein–ligand complexes.[6]

Chemicals were purchased from Sigma Aldrich (Castle Hill, NSW, Australia), except thermolysin which came from CalBiochem (San Diego, CA, USA). Mass spectrometry measurements were carried out on a Bruker Apex III, 4.7 T FT-ICR Mass Spectrometer,[21,22] fitted with an Apollo electrospray source. Thermolysin solutions were made at a concentration of 40 μM in ammonium acetate buffer (pH 6.9) by passing through a Sephadex gel filtration column (Amersham Biosciences) to remove non-volatile salts, resulting in a concentration of about 20 μM protein, with 100 μM Ca^{2+}, remaining for stability. Natural product stock solutions were prepared at a concentration of 300 μM, and small aliquots of these were added to the thermolysin, resulting in a measured concentration of between 5 and 20 μM protein, and from 5 to 120 μM natural product.

Two of the natural products, actinonin and phosphoramidon, were found from analysis by electrospray Fourier Transform Mass Spectrometry (FTMS) to bind to thermolysin (Fig. 1). Phosphoramidon was detected at 10 μM while actinonin was detected at 120 μM. At higher levels of inhibitor there was no indication of any non-specific binding.

The results were confirmed by biochemical assays. The EnzCheck® Protease assay from Invitrogen (Mount Waverley, Vic., Australia) was used to assay for protease inhibition. The assay was performed at room tem-

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Table 1. The natural products tested as inhibitors of thermolysin, and the Zincin-like metalloproteases they were known to inhibit

Natural product	Metalloprotease inhibits
Phosphoramidon	Thermolysin,[7] neprilysin,[8] elastase[9]
Bestatin	LTA-4H[10]
Actinonin	Astacin[11] stromelysin[12–14]
Aloins	MMP-8[15]
(−)-Epigallocatechin gallate	Gelatinases[16]
(−)-Epigallocatechin	Gelatinases[16]
(−)-Epicatechin gallate	Gelatinases[16]
(−)-Epicatechin	Gelatinases[16]
Glycyrrhetinic acid	Collagenase[17]
Betulinic acid	Stromelysin[18]
Bombesin	Neurolysin[19]
Kinetensin	Neurolysin[20]
Somatostatin	Neurolysin[19]
Leupeptin	Neurolysin[19]

perature in black 384-well microtitre plates with a total assay volume of 50 μl. The assay was run with concentrations of 100 μg ml^{-1} thermolysin (2.6 μM), 10 μg ml^{-1} fluorescent casein and with natural products at a concentration ranging from 10 to 0.01 mM in 10 mM Tris–HCl buffer, pH 7.8. For some assays 2% DMSO was also present, depending on the natural product. The reaction progress was monitored using a Wallac Victor 1420 multilable counter, the fluorescence excitation was 485 nm, emission 530 nm. The assay was incubated for 90 min.

The EnzCheck® Protease assay uses a casein modified to fluoresce as substrate, and on lysis of peptide bonds in the casein an increase in fluorescence signal is detected. Only the two natural products identified by mass spectrometry were found to inhibit thermolysin; however, low levels of thermolysin inhibition were detected for kinetensin.

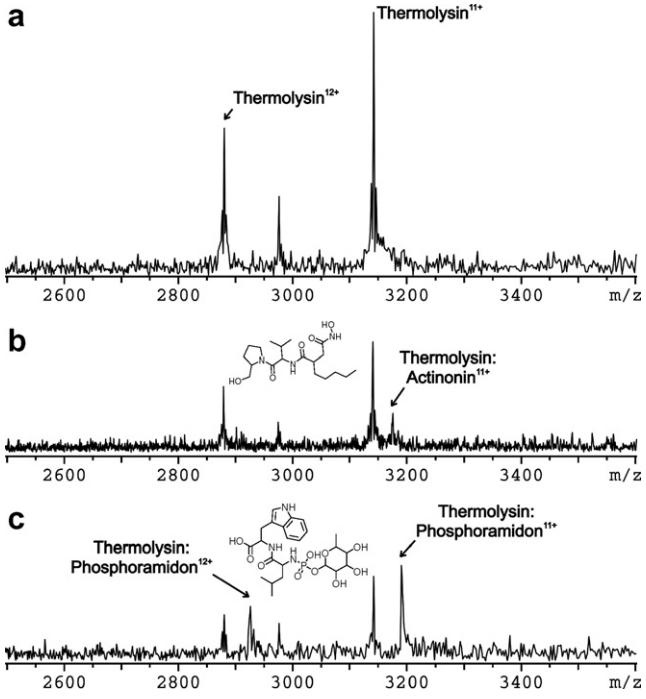

Figure 1. Mass spectra of: (a) 10 μM thermolysin; (b) 10 μM thermolysin with 120 μM actinonin present and (c) 10 μM thermolysin with 10 μM phosphoramidon present in solution. All measurements are taken in a 30 mM ammonium acetate buffer (pH 6.9), with 100 μM $CaCl_2$ present to enhance protein stability.

Thermolysin was present in the assay at a concentration of 11.7 μM giving IC_{50} values of 13.8 μM for phosphoramidon, and 18.3 μM for actinonin. An IC_{50} value could not be obtained for kinetensin. K_i was measured using the method outlined by Kitagishi[23] with *N*-(3-[2-furyl]acryloyl)-Gly-Leu amide (FAGLA) as substrate at 340 nm. The assay to measure K_i values was run with concentrations of 10 μM thermolysin, 230 μM–5.85 mM *N*-(3-[2-furyl]acryloyl)-Gly-Leu amide substrate[23] and with either phosphoramidon or actinonin at a concentration ranging from 5 nM to 10 mM in 30 mM NH_4AcO, 1 mM $CaCl_2$ buffer, pH 6.9. The assay was carried out in 384-well plates, with a reaction volume of 50 μl. The reaction progress was monitored using a Tecan Ultra, measuring the absorbance at the UV wavelength of 340 nm. The assay was incubated for 160 min at room temperature.

The K_i values for thermolysin inhibition by phosphoramidon and actinonin were measured as 6.98 and 4.88 μM, respectively. The K_i values measured for these natural products are of similar magnitude, indicating similar efficacy of inhibition by both natural products.

The affinity mass spectrometry results show a much stronger binding for phosphoramidon to thermolysin, than for actinonin. It has been shown that for some systems, K_i values measured by affinity mass spectrometry will correlate to those measured by other techniques.[24] The K_i values measured by the UV assay, however, showed that the K_i values for these two natural products binding to thermolysin were of similar magnitude. It has been shown that the degree of the retention of non-covalent complexes in electrospray mass spectrometry during the transfer from solution phase to gas phase is not equivalent for all complexes.[6] Complexes that are stabilised by ionic bonds are much more likely to be retained in the electrospray process than those stabilised by hydrophobic/hydrophilic and van de Waals interactions.[25] This observation suggests that in the gas phase the ionic force between the peptide bond in the phosphoramidon, and the binding cavity of the thermolysin, is critical to the stabilisation of the thermolysin/phosphoramidon complex. This missing polar interaction in the thermolysin/actinonin complex may be enough to de-stabilise the thermolysin/actinonin interactions in the gas phase. It follows that, in solution phase, actinonin is stabilised in the binding site of thermolysin by non-ionic interactions, which are not retained when the complex is transferred to the gas phase.

Peptide bonds were present in the structures of some of the natural products examined as potential inhibitors of thermolysin. Since the function of thermolysin is to hydrolyse a peptide bond next to a hydrophobic amino acid residue,[26] the natural products were tested to determine if they were thermolysin substrates. If this was

occurring, the natural product would still be binding to the enzyme, demonstrating the consistency of the theory linking ligand binding to protein fold type. However, natural products that are substrates of thermolysin would not be detected by either affinity mass spectrometry or fluorescence assays, since they did not remain bound to the thermolysin, or inhibit thermolysin activity.

Bestatin, bombesin, kinetensin, leupeptin and somatostatin were assayed for cleavage by thermolysin over a 180 min time period. Substrate testing was performed using solutions of 10 μM thermolysin, in 30 mM ammonium acetate (pH 6.9), and 100 μM calcium chloride. To these were added small aliquots of natural product solutions, resulting in a concentration of 60 μM natural product. Fifteen microlitre fractions were removed from the stock at a time period of 0, 60, 120 and 180 min, and the reaction in these was quenched by adding 15 μl of methanol, which served to denature the thermolysin. Each fraction was then analysed by FTMS, in order to monitor the amount of natural product remaining in the sample, and look for any new masses that may correspond to products of natural product hydrolysis.

No breakdown of bestatin, bombesin, leupeptin or somatostatin was detected. Kinetensin was found to be a substrate of thermolysin, and the level of the natural product present in the sample was completely broken down after 30 min. This finding explains the low levels of kinetensin inhibition detected in the biochemical assays.

This work has shown that actinonin is an inhibitor of thermolysin, a finding which was not previously known. Actinonin had been known to be an inhibitor of metalloproteases astacin[11] and stromelysin.[12–14] Thermolysin is a protease found in bacteria, which functions intracellularly for bacterial nutrition purposes.[4] Although astacin, stromelysin and thermolysin are all Zincin-like fold proteins, they are unrelated by function and share no significant sequence homology. In addition to these Zincin-like fold proteins, actinonin is also an inhibitor of peptide deformlyase[27] (PDF) of the Peptide Deformylase Fold.

Actinonin is available in a crystal structure complex with PDF (1g2a)[12] but not in any Zincin-like fold proteins. Docking of actinonin in thermolysin, astacin and stromelysin was undertaken to compare modes of binding within the same and different fold types. Docking was done using the program GOLD[28–31] (Genetic Optimisation of Ligand Docking) version 3.1 and standard default settings to produce 50 solutions for each experiment. Protein structures (astacin 1qji,[32] stromelysin 1hy7[33] and thermolysin 1thl[34]) were prepared by extracting the ligand, removing all waters, lone pairs and dummy atoms, and adding hydrogens. The natural product was prepared by checking atom types and bond types followed by a minimisation using the MMFF94s force field, MMFF94 charges, conjugate gradient optimisation method, and termination at a gradient of 0.05 kcal/(mol*A) without any initial optimisation. Solutions for hydrogen bonding analysis were selected on the basis of GoldScore fitness function[28,29] and Consensus Scoring.[35] Hydrogen bond interactions were analysed using a previously reported method.[1]

The docking and X-ray data are shown in Figure 2. Each of the proteins is overlaid by fold and in the case of PDF, by PFT. Figure 2 shows the spatially equivalent residues in each of the proteins (coloured equivalently) involved in hydrogen bonding with actinonin, the coordination of the ligand to the zinc and the fold around the active site.

Actinonin binding in stromelysin (Fig. 2A) and PDF (Fig. 2B) is in the same orientation with conserved hydrogen bonding and the same part of the ligand coordinated to the metal. As stromelysin and PDF belong to different folds, the recognition is at the level of PFT. Although stromelysin, astacin and thermolysin are of the same fold type, the binding of actinonin in both astacin (Fig. 2C) and thermolysin (Fig. 2D) is substantially different. This difference reflects differences in PFT namely the additional Segment II which reduces the size of the cavity. An even greater shift in the position of actinonin relative to the fold is evident in thermolysin (Fig. 2D). This is due to the reduction in size of the Loop region evident upon comparison with the other Zincin-like fold proteins and PDF.

FTMS identified actinonin as a new binding partner for thermolysin. This allowed comparison of actinonin binding in three different Zincin-like fold proteins and one Peptide Deformylase Fold protein. Docking showed

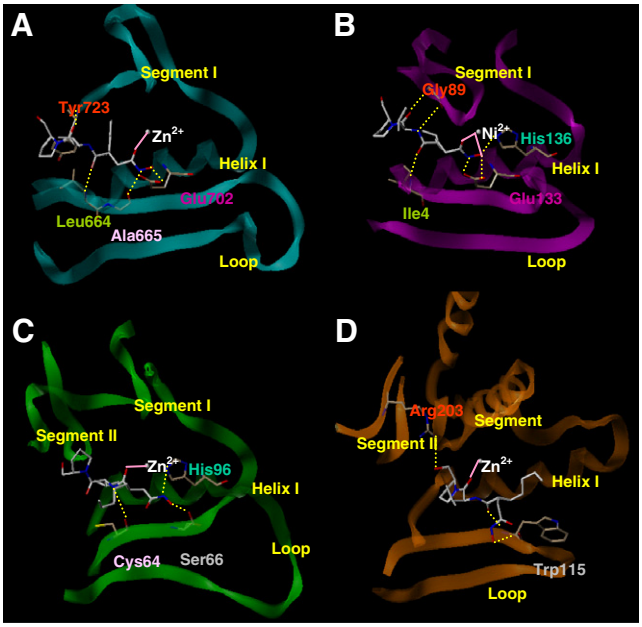

Figure 2. Actinonin docked in stromelysin (A); astacin (C); thermolysin (D) and in X-ray structure complex with PDF (B). The fold around the active site is shown in each and labelled according to PFT similarity. Spatially equivalent residues are coloured equivalently. Hydrogen bonding interactions are shown in yellow and coordination to the metal is shown in pink.

that within the Zincin-like fold, binding of actinonin is differentiated, interpretable as due to PFT recognition. The similarity in binding mode of actinonin in stromelysin and PDF reflects the shared PFT between these proteins of different fold type classifications.

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Supplementary data

Different and larger versions of Figure 2 are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.09.084.

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