Inhibition of Elastase and Trypsin
By Novel β-Lactams

By

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Abstract

Elastase is a serine protease that has been implicated in a number of inflammatory conditions including rheumatoid arthritis and cystic fibrosis. These conditions are thought to result from an increased amount of active elastase in the body, caused by insufficient inhibition by endogenous inhibitors. Elastase has the ability to degrade many tissue components and this excessive tissue damage causes the onset of a variety of inflammatory conditions. This knowledge has led to an increased interest in the production of elastase inhibitors with the hope of developing an inhibitor, which can be of therapeutic use in vivo. As a result we have synthesised a series of novel β-lactams since β-lactam compounds are known to be mechanism-based inhibitors of serine proteases. Electrospray ionisation mass spectrometry (ESI-MS) confirmed a novel mode of inhibition, for β-lactams which possess a hydroxyl group present at the C3 position, resulting in the formation of an acyl-enzyme intermediate which was found to be extremely stable. Enzyme kinetic studies demonstrated that the β-lactams had $k_{ass}$ values ranging from 1000 – 10,000 M$^{-1}$ s$^{-1}$. These β-lactams were also assayed for activity against trypsin. ESI-MS confirmed that they were also inhibitors of trypsin but enzyme kinetic studies revealed that they were more active towards elastase. Therefore the β-lactams were found to be stable, potent and specific inhibitors of elastase that may be of therapeutic use in the treatment of many inflammatory conditions.
Acknowledgements

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Special thanks to Ian for his constant support, patience and encouragement without whom it would have impossible to stay sane.

Finally I'd like to thank BBSRC for funding and making it possible to work on such an interesting subject.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>OX,-PI</td>
<td>α₁-proteinase inhibitor</td>
</tr>
<tr>
<td>O₂-M</td>
<td>α₂-macroglobulin</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-Aminoethyl) benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>Asparagine</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>E-I</td>
<td>Acyl-enzyme intermediate</td>
</tr>
<tr>
<td>EI</td>
<td>Enzyme-inhibitor complex</td>
</tr>
<tr>
<td>EI1</td>
<td>Elastase Inhibitor 1</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme-substrate complex</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray Ionisation Mass Spectrometry</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HLE</td>
<td>Human Leukocyte Elastase</td>
</tr>
<tr>
<td>Hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>I</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>k₂/Kᵢ</td>
<td>Specificity constant</td>
</tr>
<tr>
<td>kₐss</td>
<td>Apparent rate of inhibition</td>
</tr>
<tr>
<td>kₖcat</td>
<td>Turnover number</td>
</tr>
<tr>
<td>kₖcat/Kₘ</td>
<td>Specificity constant</td>
</tr>
<tr>
<td>Kₙ</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>$k_{obs}$</td>
<td>Observed rate of inactivation</td>
</tr>
<tr>
<td>$kV$</td>
<td>Kilovolts</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td>Moles</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge units</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N/A</td>
<td>Not available</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>PPE</td>
<td>Porcine Pancreatic Elastase</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed-phase liquid chromatography</td>
</tr>
<tr>
<td>S</td>
<td>Substrate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukoproteinase inhibitor</td>
</tr>
<tr>
<td>$N$-Suc-AAA-pNa</td>
<td>$N$-Succinyl-Alanine-Alanine-Alanine-para-nitroanilide</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half-life</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>v</td>
<td>Velocity</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal velocity</td>
</tr>
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1. Introduction

1.1. Serine proteases

The serine proteases are a large group of enzymes including chymotrypsin, trypsin, elastase, thrombin, plasmin and subtilisin. Serine proteases hydrolyse the peptide bonds of proteins and all share a similar structure and mechanism but differ in their specificities, which depend largely on the residues in the active site.

The serine proteases were so named, as they are dependent on a serine residue for activity. The catalytic site of each member is composed of three residues, these being serine\textsubscript{195}, histidine\textsubscript{57} and asparagine\textsubscript{102} (Bernstein et al., 1994). These three residues make up what is known as the catalytic triad. The catalytic triad is responsible for proton transfer during peptide hydrolysis.

The numbers are based on the chymotrypsin numbering system (Hartley and Kauffman, 1966). This system is the most common method of numbering since chymotrypsin was one of the first serine proteases to be fully characterised and the use of a single numbering system allows the amino acid sequences of different members to be compared.

Figure 1 shows the catalytic mechanism of serine proteases which involves the formation of a Michaelis complex (1) followed by the attack of the active-site serine hydroxyl on the carbonyl group of the substrate to form a tetrahedral intermediate which is stabilised by hydrogen bonds to glycine\textsubscript{193} and serine\textsubscript{195} (2). Collapse of the tetrahedral intermediate, with the cleavage of the amide bond results in the release of the free amine product and formation of the acyl-enzyme intermediate (3). This acyl-enzyme intermediate then undergoes hydrolysis to regenerate the enzyme and the product (4).
Figure 1: General mechanism of substrate hydrolysis by serine proteases.
In addition to the interactions that take place in the catalytic site, the substrate also interacts with the extended substrate-binding region (Figure 2). The residues which extend towards the N-terminus of the substrate are known as P1, P2, P3 etc., the residues which extend towards the C-terminus of the substrate are known as P1', P2', P3' etc and the corresponding binding subsites on the enzyme are known as S1, S2, S3, S1', S2', S3' etc (Schechter and Berger, 1967). The most important interaction in this region is the interaction between the S1 subsite and the P1 residue since this is the main determinant of substrate specificity among the serine proteases.

![Figure 2](image.png)

Figure 2: Schematic representation of a substrate in the active site of a serine protease, highlighting the protein subsites. The arrow indicates the bond that is cleaved by catalysis by the catalytic triad.

Chymotrypsin, trypsin, and elastase are the major enzymes produced by the mammalian pancreas. These enzymes have similar structures and share about 40% amino acid identity (Shotton and Hartley, 1970). Chymotrypsin has a large pocket that accommodates large hydrophobic side chains and so it prefers substrates containing phenylalanine, tyrosine or tryptophan in the S1 subsite. Trypsin has an aspartate residue at the bottom of the pocket that forms a salt bridge with the positively charged groups of its preferred substrates, lysine or arginine. The mouth of the elastase pocket is partially blocked by valine and threonine and so only accommodates small hydrophobic side chains such as alanine and valine (Shotton and Hartley, 1970).
Table 1: Table showing the P1 residues of the preferred substrates for three serine proteases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Substrate</th>
<th>Structure</th>
<th>Substrate</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td><img src="image" alt="Phenylalanine" /></td>
<td>Lysine</td>
<td><img src="image" alt="Lysine" /></td>
<td>Alanine</td>
<td><img src="image" alt="Alanine" /></td>
</tr>
<tr>
<td>Tyrosine</td>
<td><img src="image" alt="Tyrosine" /></td>
<td>Arginine</td>
<td><img src="image" alt="Arginine" /></td>
<td>Valine</td>
<td><img src="image" alt="Valine" /></td>
</tr>
<tr>
<td>Tryptophan</td>
<td><img src="image" alt="Tryptophan" /></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Serine proteases have important roles in digestion, blood coagulation, fibrinolysis, complement activation and peptide hormone activation (Neurath, 1984) therefore they are useful therapeutic targets.

Serine proteases have the ability to destroy many important protein components of cells and tissues. If proteolysis is not controlled, the onset of a wide variety of diseases can occur such as pulmonary emphysema (Snider, 1992), cystic fibrosis (Collins, 1992), rheumatoid arthritis (Barrett and Saklatvala, 1985), septic shock (Tanaka et al., 1991) and pancreatitis (Carter and Warshaw, 1989). Therefore the design and synthesis of specific inhibitors to regulate proteolysis is an extremely important area of study.
1.2. Elastase

Elastase is so called because of its unique ability to cleave the connective tissue protein elastin. Elastase can also digest other substrates such as collagen, fibrinogen and proteoglycan (Hlasta and Pagani, 1994). The two main types of elastase, which are often studied, are human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE). HLE and PPE are closely related; PPE has about 40% primary sequence homology with HLE and even greater homology in the active site. X-ray crystallographic studies have shown that HLE and PPE also share a similar structure (Edwards and Bernstein, 1994).

HLE is a 220 amino acid glycoprotein found within the primary granules of polymorphonuclear leukocytes (PMNL) or neutrophils (Edwards and Bernstein, 1994). Neutrophils are specialised phagocytic cells that mature in bone marrow and enter the bloodstream. Neutrophils are usually activated in response to inflammatory mediators such as invading microbes. Once activated the contents of the intracellular granules are released which contain a variety of proteases, of which HLE is a prime component (Vender, 1996). Therefore HLE plays a major role in the body’s inflammatory defences particularly in protein digestion following phagocytosis.

PPE is a 240 amino acid glycoprotein that is synthesized in the pancreas as an inactive zymogen known as proelastase. Proelastase is then secreted into the intestine where it’s converted to elastase by trypsin. Active elastase then participates in digestion by cleaving the peptide bonds next to small, uncharged side chains such as those of alanine and serine.

PPE is often used as model for HLE since they are structurally similar and have similar specificities but PPE is much less expensive and is readily available.
1.2.1. Role of elastase in disease

Elastase can become extremely destructive if not regulated because of its ability to destroy many connective tissue components. Elastase is normally controlled by endogenous inhibitors (Figure 3). The two main ones are $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI) and $\alpha_2$-macroglobulin ($\alpha_2$-M).

An imbalance in the ratio of the concentration of elastase to the concentration of inhibitor, in favour of elastase, has been referred to as the protease – antiprotease imbalance. This imbalance could occur due to either an increased production of elastase or by inadequate inhibition of elastase.

One reason for inadequate inhibition of elastase is that $\alpha_1$-PI is susceptible to oxidative inactivation by smoking and by the release of oxidants from macrophages at the site of inflammation (Weiss, 1989). This susceptibility to oxidative inactivation is thought to occur because the active site of $\alpha_1$-PI contains a methionine residue that is readily oxidised by agents such as hydrogen peroxide (Heidtmann and Travis, 1986).

Exposure of the lungs to tobacco smoke triggers an increase in the population of macrophages. The macrophages then release oxygen radicals, which inadvertently have the effect of inactivating all localised molecules of $\alpha_1$-PI. Some of the loss of inhibitor activity could be due to direct oxidation of $\alpha_1$-PI by nitrogen dioxide in cigarette smoke, but the main source of oxidation is thought to be from the release of oxygen radicals from macrophages (Carrell et al., 1982). In this case individuals have normal number of inhibitors but most are in an inactive form.

Another reason for inadequate inhibition is an inherited deficiency of $\alpha_1$-PI. In individuals with genetically linked $\alpha_1$-PI deficiency, the body is only able to produce a limited amount of $\alpha_1$-PI and so there is not enough inhibitor to neutralise the elastase enzyme (Crystal et al., 1989). The $\alpha_1$-PI gene is very polymorphic with 75 known alleles of which at least 11 are known to result in a deficiency of $\alpha_1$-PI. The
two most common alleles that result in α₁-PI deficiency are the Z allele and the S allele. Individuals who are homozygous for the Z allele (also known as the PiZZ variant) are at the most risk to health since these individuals only produce 15% of the normal concentration of α₁-PI. People that have S allele (the PiSS variant) produce about 60% of the normal concentrations of the inhibitor. Mixed heterozygotes will give proportionally altered plasma levels. In this case the endogenous inhibitors are active but they may be greatly reduced in numbers.

In either of these cases the activity of elastase is no longer restricted which may cause extensive tissue damage. This can lead to the onset of a variety of inflammatory diseases including cystic fibrosis, rheumatoid arthritis, emphysema, and septic shock.

Figure 3: Schematic diagram showing the regulation of elastase by endogenous inhibitors.
1.2.1.1. Cystic fibrosis

Cystic fibrosis (CF) is an inherited life-threatening disease that affects children and young adults. It is inherited in an autosomal recessive fashion so that heterozygotes that carry one normal CF allele and one mutant allele are asymptomatic and are denoted carriers. The child of two carriers then has a one in four chance of inheriting a mutation from each parent, which would result in the development of CF (Collins, 1992). The primary clinical feature of CF is the obstruction of the airways by thick, sticky mucus and bacterial infection particularly by *Pseudomonas aeruginosa*, this is known as a biofilm (Doring, 1994). Once colonization with *P. aeruginosa* has occurred, it is virtually impossible to eradicate the pathogen from the respiratory tract.

Mucoid *P. aeruginosa* forms exo-polysaccaride-coated microcolonies. These microcolonies are so large that phagocytosis is extremely difficult. The presence of the bacterium provides a chronic immunological stimulus, which causes the recruitment of large numbers of neutrophils to the airspaces of the lung. The neutrophils release huge amounts of elastase, but since the microcolonies are too large, frustrated phagocytosis occurs, which results in the release of more elastase from neutrophils (Doring, 1994). The natural inhibitor α1-PI becomes overwhelmed causing an imbalance in the protease-antiprotease ratio, in favour of the protease (elastase), which results in extensive lung damage in these patients.

Meyer and Zimmerman (1993) measured the amount of the neutrophil elastase in bronchoalveolar lavage fluid (BAL) from patients suffering from cystic fibrosis in an attempt to correlate the degree of airflow obstruction and lung bacterial burden with the level of elastase present in BAL fluid. They found that elastase activity correlated significantly with the degree of airflow obstruction but the density of *P. aeruginosa* did not (Meyer and Zimmerman, 1993). This showed that elastase was the main cause of the host inflammatory response and although *P. aeruginosa* is an important stimulus for inflammation its effect is not as great as neutrophil mediators such as elastase.
1.2.1.2. Rheumatoid arthritis

The symptoms of rheumatoid arthritis include inflammation of the joints caused by a deterioration of the cartilage. The extracellular matrix of cartilage is primarily composed of proteoglycan and collagen. Degradation of either of these structural proteins destroys the system (Barrett and Saklatvala, 1985).

Elastase can degrade cartilage proteoglycan by fragmenting the protein backbone. Neutrophil elastase has been shown to degrade cartilage proteoglycan in vitro (Moore et al., 1993). Neutrophil elastase is thought to degrade collagen by eliminating the cross-links (Barrett and Saklatvala, 1985). When the collagen component of cartilage is degraded the damage is virtually irreparable.

Moore et al., (1999) were interested in determining the role of elastase in this disease. They found that there was an excess of elastase inhibitors such as $\alpha_1$-PI and $\alpha_2$-M present in the synovial fluids, but it was not clear as to how the synovial fluids were able to degrade cartilage through an elastase dependent mechanism in the presence of active $\alpha_1$-PI. They then found that if you treat synovial fluid with a chemical, which will inactivate $\alpha_2$-M, the elastase could be fully inactivated by $\alpha_1$-PI. This suggests that $\alpha_2$-M may bind to elastase and so protect it from inactivation by $\alpha_1$-PI.

$\alpha_2$-M is thought to bind in such a way that the inhibited enzyme still regains some activity against low molecular weight substrates (1.4.1.2) (Carter and Warshaw, 1989). Therefore it is thought that is not the free elastase that is responsible for causing rheumatoid arthritis, but elastase complexed to $\alpha_2$-M (Moore et al., 1999).
1.2.1.3. Emphysema

Emphysema is characterised by enlargement of the peripheral air spaces of the lung such as the respiratory bronchioles, alveolar ducts and alveoli, accompanied by destruction of the walls of these structures (Snider, 1992). Damage is irreversible and results in permanent "holes" in the tissues. As air sacs are destroyed, the lungs are able to transfer less and less oxygen to the bloodstream, causing shortness of breath and frequently death (Massaro and Massaro, 1997).

Neutrophil elastase is thought to be the primary destructive agent in pulmonary emphysema (Powers, 1983). This is supported by the fact that emphysema can be induced in rats by administration of elastase. Massaro and Massaro (1997) instilled elastase into the lungs of rats producing changes characteristic of emphysema. These changes included increased lung volume, which indicated a loss of elastic recoil, larger, but fewer alveoli and decreased alveolar surface area due to destruction of the alveolar walls (Massaro and Massaro, 1997).

Endogenous inhibitors of elastase usually regulate the activity of the enzyme. When elastase release is excessive or when there are very little active inhibitors present an imbalance in the ratio of elastase to inhibitors occurs (1.2.1). This results in uncontrolled elastase activity, causing lung damage and the onset of emphysema.

There are two main reasons for a reduction in the number of active endogenous inhibitors. Smoking is responsible for the majority of emphysema cases as it causes oxidative inactivation of $\alpha_1$-PI. There is also an inherited form of emphysema called $\alpha_1$-antiprotease inhibitor ($\alpha_1$-PI) deficiency-related emphysema, which affects people with a deficiency of the inhibitor $\alpha_1$-PI.
1.2.1.4. Septic shock

Septic shock due to bacterial infection is characterised by inadequate blood flow in the tissues following bacterial infection of the blood. Septic shock is most common with infections by gram-negative organisms, staphylococci, or meningococci. The major symptoms of septic shock are fever, reduced urine flow, decreased alertness, confusion, and acute failure of multiple organs, particularly the lung, kidneys, and liver.

The pathogenesis of septic shock is not completely understood. The bacterial toxins produced by the bacteria trigger complex immune reactions. Since elastase is often produced in response to an immunological stimulus and is capable of degrading many tissue components, it may play an important role in the development of tissue destruction following infection in septic shock.

Multiple organ failure (MOF) frequently occurs following septic shock and neutrophil elastase has been implicated in playing a major role in tissue injury in patients with MOF (Tanaka et al., 1991). Tanaka et al., measured the elastase activity in plasma and bronchoalveolar lavage (BAL) fluid in patients with septic shock. They found an increase of elastase in the blood but it was complexed to α₁-PI and so was inactive. However the presence of active elastase in BAL fluid was significantly increased.

The results showed that elastase activity was increased locally but not systemically and so it was thought that elastase may play a role in the local destruction of inflamed tissues causing severe tissue damage in patients with septic shock and MOF.
1.3. Trypsin

Trypsin is a single chain polypeptide of 223 amino acid residues. The native form of trypsin is referred to as β-trypsin. Subsequent limited autolysis produces other active forms having two or more peptide chains bound by disulfide bonds. The predominant forms are α-trypsin and ψ-trypsin. Autolysis of β-trypsin between lysine$_{145}$ and serine$_{146}$ results in the production of α-trypsin, which has two peptide chains bound by disulfide bonds. Additional hydrolysis of α-trypsin between lysine$_{188}$ and asparagine$_{189}$ generates ψ-trypsin, which has three peptide chains joined by disulfide bonds (Chowdhury and Chait, 1990).

Trypsin is synthesized in the exocrine part of the pancreas as a zymogen. It is secreted into the pancreatic duct as an inactive form of the enzyme known as trypsinogen, which is activated by the irreversible cleavage of a hexapeptide at the N-terminal end of the molecule. Trypsinogen is converted to trypsin by enterokinase, which is secreted by the cells lining the duodenum. Once activated, trypsin is then capable of activating other molecules such as chymotrypsin, elastase, phospholipase, plasmin and thrombin (Geiger and Fritz, 1984). Since trypsin is the key point in the cascade of proteolytic activity in response to ingested food, it has an extremely important role to play in digestion (Hoerl and Heidland, 1984). As a result trypsin has several endogenous inhibitors that regulate its activity.

The crystal structure (Fehlhammer and Bode, 1975) and amino acid sequence (Shotton and Hartley, 1970) for trypsin has been solved. The active site contains the catalytic triad composed of serine, histidine and asparagine which allows the transfer of protons during peptide hydrolysis. Trypsin also has an aspartate residue at the bottom of the S1 pocket, since this aspartate residue has a negative charge, this allows the formation of a salt bridge with positively charged groups and so its preferred substrates are lysine or arginine. Site-directed mutagenesis (Evnin et al., 1990) has been carried out on trypsin to investigate its substrate specificity. It was found that negative amino acids at the 189 or 190 position at the base of the S1 pocket was essential for efficient catalysis.
1.3.1. Role of trypsin in disease

Trypsin is thought to play an important role in pancreatitis. The pancreas is a unique organ because it contains enzymes that are capable of digesting it. Trypsin is the most important of the pancreatic proteases, because once activated, it can then activate the other proteases in a self-sustaining process thereby leading to the autodigestion of the organ (Carter and Warshaw, 1989). Therefore control of the activation of trypsin has extensive consequences in terms of the formation of the components of pancreatic juice.

There are several endogenous inhibitors present in the plasma, which inhibit trypsin and so control the formation of the pancreatic juice components, these include \(\alpha_1\)-PI, \(\alpha_2\)-M and pancreatic secretory trypsin inhibitor (PSTI) (Carter and Warshaw, 1989). \(\alpha_2\)-M binds in a way that only prevents large molecules from interacting with trypsin, low molecular weight substrates can still gain access to the active site to react with trypsin (1.4.1.2).

Sobajima et al., induced the onset of acute pancreatitis by infusing trypsin into the pancreatic ductal system of rats. They then found by administering a low molecular weight synthetic inhibitor (4-(2-succinimidoethylthio)-phenyl 4-guanidinobenzoate methanesulfonate) which they called E3123, they could reduce the activity of serum \(\alpha_2\)-M complexed to trypsin (\(\alpha_2\)M-TRY). E3123 showed a reduction of the effects of the initial stage of severe acute pancreatitis and this was thought to be due to the inhibition of the \(\alpha_2\)M-TRY complex (Sobajima et al., 1994).
1.4. Inhibitors of serine proteases

A considerable effort has been aimed towards the development of inhibitors to supplement the body’s natural inhibitors in an attempt to correct the protease-antiprotease imbalance in many pathological conditions. One approach has been the use of high molecular-weight protease inhibitors, such as α₁-PI or α₂-M. Another approach has been the development of the low molecular-weight synthetic inhibitors. The use of low molecular-weight protease inhibitors has several advantages over the use of high molecular-weight protease inhibitors since synthetic inhibitors can be made which are easily absorbed orally, have greater enzyme specificity, less risk of immune response and less proteolytic inactivation (Edwards and Bernstein, 1994).

1.4.1. Endogenous inhibitors

There are a few different types of serine protease inhibitors present in the body (particularly in plasma), the most common are α₁-proteinase inhibitor (α₁-PI) and α₂-macroglobulin (α₂-M).

1.4.1.1. α₁-proteinase inhibitor

Human α₁-PI (also known as alpha₁-antitrypsin or α₁-AT) is produced in the liver, secreted into plasma and distributed to all parts of the body. It has a molecular weight of 52,000 Da and is a single polypeptide chain of 394 amino acid residues (Carrell et al., 1982).

The primary target of this protein seems to be human leukocyte elastase. There are several lines of evidence that support this:

1. A genetic deficiency of α₁-PI (Crystal et al., 1989) is associated with the development of pulmonary emphysema. This disease can be artificially induced in animals by administration of elastase (Massaro and Massaro, 1997).
2. No other disorders have been reported for α₁-PI deficient individuals, which suggest that the other serine proteases that could be controlled by α₁-PI are actually controlled by other inhibitors (Heidtmann and Travis, 1986).
3. The association rates for several serine proteases with \( \alpha_1 \)-PI have been measured (Beatty et al., 1980), and it was found that human neutrophil elastase was inhibited far more rapidly than any other protease since it had a \( k_{ass} \) of \( 10^7 \text{ M}^{-1} \text{s}^{-1} \).

The major disadvantage to the use of \( \alpha_1 \)-PI in the inhibition of elastase is that it is susceptible to oxidative inactivation due to the presence of a methionine residue in the active site. If the active site methionine residue is oxidised \( \alpha_1 \)-PI no longer has the ability to inhibit elastase. Since a major source of oxidation comes from the release of oxygen radicals from macrophages at the site of inflammation (Heidtmann and Travis, 1986), \( \alpha_1 \)-PI would have little effect on elastase present at the site of inflammation.

1.4.1.2. \( \alpha_2 \)-macroglobulin

\( \alpha_2 \)-M is a non-specific inhibitor that is synthesized by the liver, alveolar macrophages and fibroblasts (Carter and Warshaw, 1989). It has a high-molecular weight (725 000 Da) and so doesn’t readily migrate from plasma to tissue spaces.

It is exceptional in that it can inhibit enzymes from all four groups of proteases. \( \alpha_2 \)-M has four identical subunits which each have a short sequence of amino acids near the centre of the polypeptide chain, which are extremely sensitive to attack by proteases. When a protease cleaves this “bait region” it triggers a change in the conformation of the \( \alpha_2 \)-M molecule, such that the enzyme becomes physically trapped within it. Since the \( \alpha_2 \)-M molecule doesn’t bind to the enzyme at its active site the enzyme remains reactive to small molecular weight compounds that are able to diffuse into the interior of the trap (Barrett and Starkey, 1973).

Therefore \( \alpha_2 \)-M is also not an ideal candidate for inhibiting elastase since it is extremely non-specific and allows elastase to regain activity against low molecular weight substrates.
1.4.2. Synthetic inhibitors

1.4.2.1. Reversible inhibitors

Peptide aldehydes

Peptide aldehydes are known to be efficient reversible inhibitors of serine proteases (Thompson, 1973). Optimisation of the peptide backbone of these aldehydes has resulted in very potent inhibitors. The mechanism is thought to involve covalent attack of the aldehyde carbonyl carbon atom on the active site serine hydroxyl group. Elastatinal (Figure 4) and leupeptin (Figure 5) are aldehyde inhibitors that are specific to elastase and trypsin respectively (Edwards and Bernstein, 1994). Elastatinal inhibits PPE with a $K_i$ of 0.2 μM (Feinstein, 1976) whereas leupeptin inhibits trypsin with a $K_i$ of 0.13 μM (Kuramochi et al., 1979).

![Figure 4: Structure of Elastatinal (N-[[(S)-1-carboxy-isopentyl]-carbamoyl-α-(2-iminohexahydro-4(S)-pyrimidyl]-L-glycyl-L-glutaminyl-L-alaninal)].](image)

![Figure 5: Structure of Leupeptin (N-acetyl-L-leucine-L-leucine-L-argininal)].(image)
Peptidyl trifluromethyl ketones

Peptidyl trifluromethyl ketones (TFMKs) are known to be 10-100 fold more potent than the corresponding aldehyde (Edwards and Bernstein, 1994). TFMKs inhibit using a similar mechanism to the aldehydes. They are known to be tight-binding inhibitors of elastase with very slow off-rates. Williams et al., (1991) synthesized a peptidyl TFMK known as ICI 200,880 (Figure 6), which was shown to be a selective, potent slow-binding PPE inhibitor with a $K_i$ of 0.5 nM (Williams et al., 1991).

![Figure 6: Structure of ICI 200,800 (4-(4-chlorophenylsulfonycarbamoyl) benzyol-L-valinyl-L-prolyl-1-(RS)-(1-trifluoroacetly-2-methylprolyl)amide)].

1.4.2.2. Irreversible inhibitors

Peptidyl hydroxymates

Peptidyl hydroxymates are a class of irreversible inhibitors of serine proteases. These compounds allow variations in the N-acyl and O-acyl residues and so the affinity and reactivity towards the target enzyme can be regulated. The mechanism involves carbamylation of the active site serine, after rearrangement of the inhibitor molecule (Steinmetz et al., 1994). One such inhibitor of PPE is Boc-Ala-Ala-Ala-NHO-Bz known as elastase inhibitor 1 (EI1) (Figure 7). This compound inhibits elastase with a $k_2/K_i$ of $128 \text{ M}^{-1} \text{ s}^{-1}$ (Schmidt et al., 1991). The mechanism of elastase inhibition by EI1 is shown in Figure 47.
Sulphonyl fluorides

Sulphonyl fluorides are well known irreversible inhibitors of serine proteases. Most of these types of compounds react quite slowly with proteases. They react with the active site serine to form a sulfonyl ester. 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) (Figure 8) is an example of this type of inhibitor that irreversibly inhibits trypsin (Markwardt et al., 1974).

Coumarins

Isocoumarins are specific, mechanism-based, heterocyclic inhibitors of serine proteases. Proteases react with these compounds to cleave the heterocyclic ring they then form an intermediate acyl-enzyme and this new species then alkylates a second active site residue to permanently inactivate the enzyme (Edwards and Bernstein, 1994). Figure 9 summarises the mechanism of inhibition of the 3-chloroisocoumarins.
3-chloroisocoumarin (Figure 10) is an inhibitor of elastase that has a $k_{\text{ass}}$ value of $512 \, \text{M}^{-1} \, \text{s}^{-1}$.

**β-lactams**

β-lactams are perhaps most commonly known for their antibiotic properties. Soon after the discovery of penicillin (Figure 11) it was found that bacteria were able to produce β-lactamases that could hydrolyse the β-lactam to its corresponding amino acid (Laws et al., 1993).
Compounds were then developed which themselves had no antibiotic properties but possessed the ability to inhibit bacterial β-lactamases. These compounds could then be administered with the antibiotic, to inhibit the β-lactamases produced by the bacteria leaving the antibiotic active and able to destroy the bacteria. One of the first of these inhibitors to be discovered was clavulanic acid (Figure 12). This compound was found to be a very potent inhibitor of β-lactamase and is now commonly administered alongside antibiotic drugs such as amoxyccillin (Nicolas-Chanoine, 1997).

![Figure 12: Structure of clavulanic acid.](image)

Due to its clinical importance clavulanic acid has been extensively studied and its mechanism of action has been elucidated (Imtiaz et al., 1993). It was soon discovered by the Merck group that β-lactam containing compounds were also potent inhibitors of others members of the serine proteases family, particularly elastase (Knight et al., 1992). Original work concentrated on the development of substituted cephalosporin derivatives (Doherty et al., 1986), (Finke et al., 1990), (Doherty et al., 1990). One of these types of HLE inhibitors was a cephalosporin derivative (Figure 13), which had a $k_{ass}$ of 161,000 M$^{-1}$ s$^{-1}$ (Doherty et al., 1990).

![Figure 13: Structure of a cephalosporin derivative.](image)
Cephalosporin esters were soon found to be unstable, since they have short half-lives, typically 5 hours (Edwards and Bernstein, 1994). Since this instability limits their clinical usefulness, compounds were needed which were much more stable. This led to the development of monocyclic β-lactams (Figure 14) that were found to be more stable than cephalosporin esters.

![Figure 14: Basic structure of a monocyclic β-lactam.](image)

One such monocyclic β-lactam, which was also developed by the Merck group, was L-680,861 (Figure 15), which has a $k_2/K_1$ of 867,000 M$^{-1}$ s$^{-1}$ (Knight et al., 1992).

![Figure 15: Structure of L-680,861](image)
Elastase inhibition by other researchers

One of the major research groups studying the inhibition of elastase by synthetic inhibitors are located in the University of Oxford’s centre for molecular sciences and among others its researchers include R T Aplin, C J Schofield, N J Westwood and R C Wilmouth.

Wilmouth *et al.*, used ESI-MS, NMR and X-ray crystallography to identify the inhibition of elastase by a series of *N*-sulfonylaryl β-lactams (Figure 16). The studies on these compounds showed that there was no cleavage of the sulphonamide bond during inhibition. It was also found that stereochemistry was extremely important since compounds based on structure 2 were better inhibitors than compounds based on structure 1. The proposed mechanism involves the initial noncovalent binding to PPE. In order for this to occur the C3 group must be located in the S1 subsite and the ester carbonyl in the oxyanion hole. Formation of a tetrahedral complex would then result in an acyl-enzyme complex. Water can bind to this complex causing hydrolysis with the release of a β-amino acid. Alternatively a conformational change in which the ester carbonyl rotates out of the oxyanion hole can occur.

![Figure 16: General structures of the *N*-sulfonylaryl β-lactams.](image)

This group also identified a general method for inhibiting serine enzymes by analysing the reaction of several clavams (Figure 17) with elastase (Wilmouth *et al.*, 2000). They proposed that a possible mechanism for the inhibition of PPE by
esterified derivatives of clavulanic acid involves the hydrolysis of the intermediate imine by a water molecule within the active site to generate an aldehyde.

Figure 17: General structure of clavams.

The importance of pH when analysing enzyme inhibition has also been demonstrated by this group (Wright et al., 2000). Elastase was allowed to react with a γ-lactam and crystals were prepared. These crystals were then placed in a buffer of increased pH in order to subject them to ‘pH-jumps’. The results show that the conformation of the γ-lactam-derived acyl-enzyme species in the active site of PPE is dependant of pH.

Recently Wilmouth et al. (2001), identified the X-ray crystal structure of a tetrahedral intermediate formed during the hydrolysis of an acyl-enzyme complex formed between a natural heptapeptide and elastase. Peptide hydrolysis by serine proteases proceeds via the formation of an initial noncovalent enzyme-substrate complex. Nucleophilic attack by the active site serine of the peptide carbonyl results in the formation of a tetrahedral oxyanion intermediate which collapses to form an acyl-enzyme intermediate. Subsequent hydrolytic attack by a water molecule of the ester carbonyl leads to the second tetrahedral intermediate, which collapses to give the N-terminal product fragment. Wilmouth et al., identified structures for the intermediates in this reaction by flash freezing each crystal in liquid nitrogen after the reaction was allowed to proceed for a given time period.

The work presented in this thesis investigates the inhibitory effect of novel monocyclic β-lactams (similar to the ones developed by Merck) on elastase, in order to develop an effective elastase inhibitor that may be used in the treatment of a variety of diseases.
1.5. **UV-visible spectrosocpy**

UV-visible spectrophotometers have two light sources, usually a deuterium lamp for UV measurements (160 – 375 nm) and a tungsten lamp for visible measurements (350 –2500 nm). This source light is then focused on the sample using a lens in order to maximise the light intensity. Focusing is important in spectrophotometers that use diode array detectors because each individual detector in the diode array must receive enough light to make its own absorbance measurement. If a diode array has 1000 detectors, the light intensity at each one is only 1/1000 of the original source intensity. After the focused beam passes through the sample, a diffraction grating is used to disperse the light into its component wavelengths and reflect light to the diode array detector. The current at each diode is measured to make a complete spectrum (Figure 18).

![Figure 18: UV-Visible Spectrophotometer with a diode array detector.](image)

When an enzyme acts on a substrate whose product absorbs at a different wavelength from the original substrate, this change in absorbance can be detected and measured by the UV-visible spectrophotometer. A variety of synthetic colorimetric substrates are now available which allow the kinetics of most of the well-characterised enzymes to be studied.
1.5.1. Enzyme kinetics

Substrate Kinetics

Monitoring the change in absorption over time allows the calculation of the initial rate of the enzyme, and by repeating the assay with increasing concentrations of substrate, various kinetic parameters can be calculated (Lehninger et al., 1993).

Perhaps one of the most useful kinetic constant is the Michaelis constant \( (K_m) \) which is defined as the concentration of substrate at which the velocity is half the maximum rate. \( K_m \) is a measure of how tight the substrate binds to the enzyme. A low value for \( K_m \) indicates a tight binding substrate whereas a high value for \( K_m \) indicates a looser binding of substrate, which does not fit as well into the enzymes active site (Mathews and VanHolde, 1996).

\( V_{\text{max}} \) is another parameter that is often calculated, this refers to the maximal velocity of which the enzyme achieves under optimal conditions (Mathews and VanHolde, 1996).

\( k_{\text{cat}} \) is the rate of the breakdown of the enzyme-substrate complex to form the product and the free enzyme. In simple systems like Equation 1, \( k_{\text{cat}} = k_2 \). When expressed as moles of substrate consumed per unit of time, \( k_{\text{cat}} \) is often referred to as the turnover number. A high value for \( k_{\text{cat}} \) indicates efficient catalysis (Mathews and VanHolde, 1996).

Another particularly useful constant, which can be calculated, is the specificity constant \( (k_{\text{cat}}/K_m) \). \( k_{\text{cat}}/K_m \) represents the binding and catalysis of the enzyme and the substrate and is often used to compare the substrate specificity for a variety of enzymes. The higher the value for \( k_{\text{cat}}/K_m \) the more specific the substrate is for the particular enzyme (Mathews and VanHolde, 1996).

It is now well established that the basic mechanism of enzyme-catalysed substrate hydrolysis (Equation 1) (Lehninger et al., 1993) is binding of the enzyme \( (E) \) to the
substrate (S) to form a reversible complex known as the enzyme-substrate complex (ES) which has a dissociation constant $K_d (K_d = \frac{k_{-1}}{k_1})$. This complex then breaks down by a first-order process with a rate constant of $k_2$ to release the free enzyme and product. $K_m$ is analogous to $K_d$ when dissociation is much more rapid than conversion to product e.g. $k_{-1} \gg k_2$. In general $K_m = (k_2 + k_{-1}) / k_1$.

**Equation 1:** Basic mechanism of substrate hydrolysis. The enzyme (E) and substrate (S) form a reversible complex known as the enzyme-substrate complex (ES), which breaks down to produce the free enzyme (E) and product (P).

$$
E + S \quad \overset{k_1}{\underset{k_{-1}}{\leftrightarrow}} \quad ES \quad \overset{k_2}{\rightarrow} \quad E + P
$$

The Michaelis-Menten equation (Equation 2) is a quantitative description of the relationship between the rate of the enzyme-catalysed reaction ($v$), concentration of substrate [$S$], the maximal velocity of the reaction ($V_{\text{max}}$) and the Michaelis-Menten constant ($K_m$) (Mathews and VanHolde, 1996).

**Equation 2:** Michaelis-Menten equation. $V_{\text{max}}$ is the maximal velocity, [$S$] is the substrate concentration and $K_m$ is the Michaelis constant.

$$
v = \frac{V_{\text{max}} [S]}{K_m + [S]}
$$

Graphical analysis (Figure 19) of the initial reaction rate ($v$) against the substrate concentration [$S$] produces a hyperbolic rate plot. The velocity approaches $V_{\text{max}}$ at high substrate concentrations (shown by C on Figure 19) when the enzyme is saturated with substrate and the reaction becomes independent of substrate concentration. At low substrate concentrations (shown by A on Figure 19) only a
portion of the enzyme molecules are bound to substrate. At this point the velocity is
directly proportional to the substrate concentration. At substrate concentrations
denoted by B exactly half of the enzyme molecules are bound to the substrate and so
the rate is $\frac{1}{2} V_{\text{max}}$ and the concentration of substrate at this point is the Michaelis
constant ($K_m$). (Mathews and VanHolde, 1996). $k_{\text{cat}}$ can also be determined from
this plot if the total enzyme concentration $[E_T]$ is known since $k_{\text{cat}} = \frac{V_{\text{max}}}{[E_T]}$.

![Figure 19: Dependence of velocity of an enzyme-catalysed reaction on substrate concentration. A is used to show low substrate concentration, B shows the concentration of substrate at which the velocity is at $\frac{1}{2} V_{\text{max}} (K_m)$ and C indicates substrate saturation.](image)

In order to avoid dealing with hyperbolic plots it is common to replot the data in
linear form. One of the most common is the double-reciprocal Lineweaver-Burk plot
(Lineweaver and Burk, 1934), in which the Michaelis-Menten equation is rearranged
to $\frac{1}{v} = \frac{1}{V_{\text{max}}} S + \frac{1}{V_{\text{max}}}$]. Plots of $1/v$ versus $1/[S]$ give straight lines
with a slope of $K_m/V_{\text{max}}$, the intercept on the y-axis $= 1/V_{\text{max}}$ and the intercept on the
x-axis yields $-1/K_m$. A more accurate linear transformation is the Eadie-Hofstee
transformation in which the Michaelis-Menten equation is rearranged to form $v/[S] =$
\[-v \left( \frac{1}{K_m} \right) + \left( \frac{V_{max}}{K_m} \right) \]. In this case \(v[S]\) is plotted on the y-axis against \(v\) on the x-axis, this linear plot has a slope of \(-1/K_m\), the intercept on the y-axis is \(V_{max}/K_m\) and the intercept on the x-axis is \(V_{max}\) (Mathews and VanHolde, 1996).

Therefore by measuring the initial rate of the enzyme in the presence of increasing concentrations of substrate, many kinetic parameters can be obtained which give important information about the enzyme.

Table 2: Summary of the main kinetic parameters associated with substrate kinetics.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Synonym</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1)</td>
<td></td>
<td>Association rate constant</td>
</tr>
<tr>
<td>(k_{-1})</td>
<td>(k_{cat})</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>(k_2)</td>
<td>(K_m = k_{cat}/k_{-1})</td>
<td>Turnover number</td>
</tr>
<tr>
<td>(K_m)</td>
<td>(K_m = k_{-1}/k_{-1})</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>(k_{cat}/K_m)</td>
<td></td>
<td>Specificity constant</td>
</tr>
</tbody>
</table>

Inhibition kinetics

In the same manner that measuring the activity of an enzyme can yield a lot of information about how it binds to a substrate, measuring the activity of an enzyme after it has been incubated with an inhibitor can tell a lot about the inhibition process. Inhibitors generally fall into two broad categories, reversible inhibitors & irreversible ones.

Reversible Inhibition

Reversible inhibitors normally bind to the enzyme using non-covalent bonds such as hydrogen bonding. The simplest mechanism of reversible inhibition (Equation 3) is when the enzyme (E) and inhibitor (I) are in equilibrium with the enzyme-inhibitor complex (EI) (Salvesen and Nagase, 1989). The rate of inhibition is determined by the association rate constant \((k_1)\), whereas the reverse reaction is determined by the dissociation rate constant \((k_{-1})\). The inhibition constant \((K_i)\) represents the affinity of
the enzyme for the inhibitor. If the rate of dissociation is much slower than association the reverse reaction may be negligible and inhibition may be considered as irreversible.

\[
\begin{align*}
E + I & \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \quad EI
\end{align*}
\]

Equation 3: Basic mechanism of reversible inhibition. The enzyme (E) and Inhibitor (I) form a reversible enzyme-inhibitor complex (EI).

For reversible inhibitors the most useful kinetic parameter is \( K_i \), which is analogous to \( K_m \) in substrate kinetics, and so reflects the affinity of the enzyme for the inhibitor. In most cases the \( K_i \) can be calculated in the manner as \( K_m \). The residual activity of the enzyme is measured after incubation with inhibitor. This is then repeated using increasing concentrations of inhibitor (Figure 20). The data is then replotted in linear form to obtain the \( K_i \). The \( K_i \) is determined from either the intercept on the x-axis of a lineweaver-burk plot, which is \(-1/K_i\), or from the slope of an Eadie-Hofstee plot, which is also \(-1/K_i\) (Mathews and VanHolde, 1996).

Figure 20: Effect of increasing concentrations of inhibitor on initial rate.
This data can also yield some useful information about the type of reversible inhibition. By comparing the values of $K_m$ and $V_{\text{max}}$ (Table 3) obtained in a control experiment, in which no inhibitor was added, to those obtained from the inhibited samples it is possible to determine whether the inhibitor is competitive, uncompetitive or a non-competitive one (Mathews and VanHolde, 1996).

### Table 3: Main types of reversible inhibition and their effect on $K_m$ and $V_{\text{max}}$

<table>
<thead>
<tr>
<th>Type of inhibition</th>
<th>Effect on $K_m$</th>
<th>Effect on $V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
<tr>
<td>Non-competitive</td>
<td>No change</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Competitive inhibition is the most common type of reversible inhibition. The inhibitor competes for the same binding site on the enzyme as the substrate, therefore by adding an excess of substrate the effect of the inhibitor can be overcome. The $V_{\text{max}}$ can still be reached in spite of the presence of inhibitor at high substrate concentrations and so remains unchanged. Competitive inhibitors cause an increase in $K_m$ since the $K_m$ is a measure of the enzyme-substrate affinity. In the reaction mixture the enzyme will exist in one of two forms, as free enzyme molecules or as enzyme-inhibitor complexes. The substrate can only bind to the free enzyme molecules, which will have normal affinity for the substrate. The enzyme molecules, which are already bound to inhibitor, have no affinity for the substrate. $K_m$ is a measure of the overall affinity of the enzyme in the reaction mixture and since only a portion of the enzyme molecules are available for substrate binding a higher number of substrate molecules than normal are required to achieve the half-maximal velocity and so the $K_m$ is increased (Mathews and VanHolde, 1996).

Uncompetitive inhibitors are much less common and can only bind to the enzyme substrate complex, not the free enzyme itself. These inhibitors are inactive at low substrate concentrations since most of the enzyme is in the free form. They are active at higher substrate concentrations when most of the enzyme is in the enzyme-
substrate form. Since the inhibitor is most effective at high substrate concentrations it has the effect of decreasing the $V_{\text{max}}$. These inhibitors reduce the concentration of the enzyme-substrate complex when they bind to them, changing them into an enzyme-inhibitor-substrate complex, this has the effect of shifting the equilibrium to the right and so increasing the amount of substrate binding to the enzyme. This results in an increase in enzyme-substrate affinity and so a decrease in the $K_m$ occurs (Mathews and VanHolde, 1996).

Non-competitive inhibitors bind to the enzyme at a different site from that of the substrate. Therefore it has no effect on enzyme-substrate binding and so the $K_m$ remains unchanged. However binding of the inhibitor usually causes a conformational change in the enzyme such that the enzyme can no longer convert the substrate into product. These inhibitors work even at high substrate concentrations and so have the effect of decreasing the $V_{\text{max}}$ (Mathews and VanHolde, 1996).

**Irreversible inhibition**

Irreversible inhibitors normally bind covalently to the enzyme. One of the most common mechanisms of irreversible inhibition (Equation 4) is where the enzyme (E) and inhibitor (I) initially form a reversible enzyme-inhibitor complex (EI). Thereafter an irreversible enzyme intermediate (E-I) is formed for which the rate constant is termed $k_2$ or $k_{\text{inact}}$. The enzyme intermediate then breaks down to yield the free enzyme and the modified inhibitor. The rate constant for this step is known as $k_3$ or $k_{\text{react}}$. In more complicated schemes there can be many steps, which occur after the formation of the acyl-enzyme and before the release of the free enzyme and modified inhibitor (Knight et al., 1992).
Equation 4: Basic mechanism of irreversible inhibition. The enzyme (E) and Inhibitor (I) initially form a reversible enzyme-inhibitor complex (EI). This complex then forms an enzyme intermediate (E-I), which breaks down to release the free enzyme (E) and the modified inhibitor (I').

Most irreversible inhibitors do not inhibit the enzyme indefinitely but are so called because the inhibitor is irreversibly modified during catalysis.

The most useful kinetic parameter associated with irreversible inhibitors is probably $k_{ass}$, which is the apparent rate of inhibition (or association). The meaning of $k_{ass}$ is given by the relationship $k_{ass} = (k_1 \times k_2)/k_{-1}$ (Salvesen and Nagase, 1989). The value for $k_{ass}$ can be calculated from the relationship $k_{ass} = k_{obs}/[I]$ (Salvesen and Nagase, 1989). $k_{obs}$ is the observed rate of inactivation (pseudo-first-order rate constant) and can be calculated by measuring the residual activity of the enzyme after it has been pre-incubated for set period of time with increasing concentrations of inhibitor (Salvesen and Nagase, 1989). The log (ln) of the residual activity (a) can be plotted (Figure 21) against time (t) according to the equation $\ln a_t = -k_{obs}t$. $k_{obs}$ can then be obtained from the slope of the line (Knight, 1986).
Figure 21: Effect of time on the activity of enzyme incubated with an irreversible inhibitor.

$K_i$ and $k_2$ can be obtained from the secondary plots of $1/k_{obs}$ versus $1/[I]$ (Knight, 1986), the straight-line plot (Lineweaver-Burk) has an intercept on the y-axis of $1/k_2$ and the intercept on the x-axis is $-1/K_i$. If the values for $K_i$ and $k_2$ are known then the value for the specificity constant can be determined. The specificity constant ($k_2/K_i$) is a measure of the overall efficiency of the enzyme inhibition, which is analogous to $k_{cat}/K_m$ in substrate kinetics.

Another useful parameter when measuring the kinetics of irreversible inhibitors is the half-life ($t_{1/2}$) which is the time required for the free enzyme concentration to decrease by 50%, and is given by $t_{1/2} = 0.693/k_{obs}[I]$ (Henderson, 1972). This relationship can be used to compare the inactivation of an enzyme by a variety of irreversible inhibitors.
Table 4: Summary of the main kinetic parameters associated with Inhibition.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Synonym</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td></td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td></td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$k_{\text{inact}}$</td>
<td>Turnover number</td>
</tr>
<tr>
<td>$K_i$</td>
<td>$K_i = k_{-1}/k_1$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>$k_{2}/K_i$</td>
<td>$k_{\text{inact}}/K_i$</td>
<td>Specificity constant</td>
</tr>
<tr>
<td>$k_{\text{ass}}$</td>
<td>$k_{\text{ass}} = (k_1 \times k_2)/k_{-1}$ or $k_{\text{obs}}[I]$</td>
<td>Apparent rate of inhibition (association)</td>
</tr>
<tr>
<td>$k_{\text{obs}}$</td>
<td></td>
<td>Observed rate of inactivation</td>
</tr>
<tr>
<td>$t_{1/2}$ (half-life)</td>
<td>$0.693/k_{\text{ass}}[I]$</td>
<td>Time required for the free enzyme to decrease by 50%</td>
</tr>
</tbody>
</table>
1.6. Electrospray ionisation mass spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) is an extremely useful tool in the study of protein interactions. It is particularly useful in the study of enzyme inhibition since it allows the detection of intermediates that can provide evidence for the mechanism of inhibition.

ESI-MS is especially useful for the study of high molecular weight proteins because it produces sample ions that carry multiple charges that are then detected on the mass/charge (m/z) scale. A mass spectrometer, which can detect up to 3,000 on the m/z scale, can only detect singly charged molecules up to 3,000 Da, but if the molecules had multiple charges then compounds with much higher molecular weights can be analysed. For example if a protein with a molecular weight of 100,000 Da has 100 charges, it would be detected on the m/z scale at 1001.0 (Ashton et al., 1994). Therefore so long as the sample has enough protons ESI-MS can detect compounds with infinite mass. This was a major breakthrough in the study of high molecular weight proteins.

1.6.1. Instrumentation

Figure 22 shows the main steps in the process of ESI-MS. The sample is normally prepared in a volatile solvent such as 50% acetonitrile (MeCN) or methanol. The sample solution is sprayed from a fine stainless steel capillary, flowing at 2-20 μl/min. For positive ion formation a voltage of about 4-5 kV is applied to the tip of the capillary. This generates a strong electric field, which causes the solvent to be sprayed as a mist of small droplets, which then vaporise as they enter the heated source region. This vaporisation is helped by a flow of warm nitrogen gas. The density of the electrical charge builds up until a point of instability is reached. Residual sample ions start to desorb from the droplets surface, once the ions are in the gas phase they enter the vacuum of the mass spectrometer where they are separated and detected according to their m/z ratio (Mann and Wilm, 1995).
Figure 22: Schematic representation of the main steps of ESI-MS. The sample is introduced through the inlet and enters the source where the sample vaporises and forms ions. The ions are sorted in the analyser before they enter the detector. The data is analysed and results are recorded.

Figure 23 shows the mass spectrum of PPE; charges +9 to +20 are clearly visible. Each of these signals can be used to determine the molecular weight of the sample according the equation \( m/z = (MW + nH)/n \), this equation can be rearranged to \( MW = (m/z - H) \times n \), where \( MW \) is the molecular weight, \( m/z \) is the value on the mass/charge scale, \( n \) is the number of charges and \( H \) represents a proton which has a mass of 1.00794 Da (Mann et al., 1989). According to Figure 23 the largest peak at about 1439.65 m/z has 18 charges so the apparent mass of the molecule is \((1439.65 - 1.00794) \times 18 = 25895.6 \) Da. The calculated molecular weight of this enzyme is 25898.1 Da (Aplin et al., 1992). Computer software such as Mass lynx can automatically perform this calculation on each peak and then calculate the average mass based on the data to yield a deconvoluted spectrum such as in Figure 24.
Figure 23: LC-MS spectra of 100 μM of PPE.

Figure 24: Deconvoluted Mass spectra of 100 μM of PPE.
1.6.2. Detection of covalent species

Covalent inhibitors can easily be detected by liquid chromatography mass spectrometry (LC/MS) since they remain bound to the enzyme even after liquid chromatography. The covalent bond will also remain even if the enzyme has become denatured, therefore a wide variety of organic solvents may be used during LC/MS. The use of liquid chromatography prior to mass spectrometry allows the removal of buffers, unbound inhibitor or other contaminants.

Reversed-phased liquid chromatography (RP-LC) of proteins is often employed. This usually involves the sample passing through a hydrophobic silica-based column. The protein binds to the column while the impurities or other components which are not of interest, pass straight through. The mobile phase is a mixture of water and a miscible organic solvent such as acetonitrile, methanol or propanol. The protein is then eluted by gradient elution (Corran, 1998). Although RP-LC purifies the sample, because the inhibitor binds covalently to the enzyme, they do not dissociate.

After RP-LC the purified inhibited enzyme enters the mass spectrometer. The data is deconvoluted and a peak should be observed corresponding to the mass of the enzyme. If inhibition has occurred then an additional peak should also be visible which reflects the enzyme covalently bound to the inhibitor.

The difference in mass between the native enzyme peak and the inhibited enzyme peak reflects the size of the inhibitor after it has reacted with the enzyme. This mass can help determine the mechanism of inhibition, for example if an inhibitor is thought to react by forming an intermediate with a mass of 410 Da and a peak is observed with a mass shift of 410 then this provides evidence for the mechanism of inhibition.
1.6.3. Detection of non-covalent species

LC/MS is not possible if you want to detect non-covalent inhibition because the protein must remain in its native conformation to maintain non-covalent interactions with the inhibitor. The organic solvents involved in LC-MS denature the enzyme causing the enzyme and the noncovalent inhibitor to dissociate from one another. Therefore direct infusion into the mass spectrometer is necessary.

The enzyme should be purified as much as possible beforehand taking care to maintain the activity of the enzyme. Samples should be prepared in buffers at the optimum pH of the enzyme (Loo, 1997), the most common buffer used is ammonium acetate buffer. In LC/MS high flow rates are often used for convenience. In order to vaporise the molecules as they come into the instrument at these high speeds, high source temperatures are required (about 140 °C). These high temperatures would denature the enzyme and so when attempting to detect non-covalent species, low source temperatures of below 40 °C should be used (Loo, 1997). Since the source temperature is low the enzyme should then be infused into the instrument using low flow rates (about 2 μl/min) so that the entire sample is quickly vaporised (Loo, 1997).

Another factor to consider is the cone voltage. A sample cone voltage between 25V and 70V will produce ions from most samples. Normally the aim is to adjust the cone voltage until you find the maximum intensity of the peaks. For proteins of 25,000 Da this value will be around 70V whereas for low molecular weight compounds of only a few hundred Da this will be 25V. The higher the cone voltage is, the higher the chance of denaturation and fragmentation of the protein. Therefore when analysing non-covalently bound complexes a cone voltage of around 40V is preferable (Loo, 1997).
2. Experimental Methods

2.1. Elastase

2.1.1. Materials

Porcine Pancreatic Elastase (PPE) and the known covalent elastase inhibitor \( N\text{-}L\text{-}Boc\text{-}L\text{-}Ala\text{-}L\text{-}Ala\text{-}L\text{-}Ala\text{-}NHO\text{-}Bz \) (Elastase Inhibitor 1 or EI1) was purchased from Calbiochem Co. The colorimetric substrate \( N\text{-}Succinyl\text{-}Alanine\text{-}Alanine\text{-}Alanine\text{-}Alanine\text{-}para\text{-}nitroanilide \) (\( N\text{-}Suc\text{-}AAA\text{-}pNa \)) and the known non-covalent elastase inhibitor elastatinal was purchased from Sigma. Novel \( \beta \)-lactams (Table 5) were synthesised by Dr James Dowden and Kevin Bailey from the University of Edinburgh and we all single diasteromers. All other reagents and chemicals were purchased from sigma unless otherwise stated.

![Figure 25: Structure of Elastatinal \((N\text{-}[(S)\text{-}1\text{-carboxy\text{-}isopentyl})\text{-}carbamoyl\text{-}\alpha\text{-}(2\text{-iminohexahydr})\text{-}4\text{(S)\text{-}pyrimidyl})\text{-}L\text{-}glycyl\text{-}L\text{-}glutaminy}\text{-}L\text{-}alaninal)\).](image1)

![Figure 26: The structure of EI1 \((N\text{-}Boc\text{-}L\text{-}Ala\text{-}L\text{-}Ala\text{-}L\text{-}Ala\text{-}NHO\text{-}Bz)\).](image2)
<table>
<thead>
<tr>
<th>Name β-lactam</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td><img src="image1.png" alt="Structure of JD260" /></td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image2.png" alt="Structure of JD261" /></td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image3.png" alt="Structure of JD263" /></td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image4.png" alt="Structure of JD264" /></td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image5.png" alt="Structure of JD417" /></td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image6.png" alt="Structure of JD420" /></td>
</tr>
<tr>
<td>JDx1</td>
<td><img src="image7.png" alt="Structure of JDx1" /></td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image8.png" alt="Structure of JDx3" /></td>
</tr>
</tbody>
</table>
2.1.2. Preparation of stock solutions and buffers

Stock solutions of PPE (100 µM) were prepared by dissolving 0.0026 g in 1 ml of Ammonium Acetate Buffer. PPE was stored at 4 °C since the freeze thawing process was found to be detrimental to the enzyme activity. The ammonium acetate buffer (2.5 mM) was prepared by dissolving 0.096 g of ammonium acetate in 500 mls of deionised water, the pH was set to 8.0. Tris buffer (50 mM) was prepared by dissolving 0.6 g of tris base in 100 mls of deionised water and the pH was set to 8.0. Stock solutions (100 mM) of the substrate N-Suc-AAA-pNa were prepared by dissolving 0.2257 g in 5 ml of dimethylsulfoxide (DMSO). Stock solutions of 10 mM Elastatinal, Elastase Inhibitor 1, and the β-lactam compounds were stored in 1 ml of DMSO (~ 4 mg/ml depending on MW). If more dilute concentrations of these stock solutions were required then serial dilutions were carried out until the required concentration was obtained.

2.1.3. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Payne, 1976) is a method of determining the molecular weight of a protein by treating with SDS, an anionic detergent which binds to proteins based on their mass and dominates their electric charges. Electrophoresis then separates the proteins based on the extent of the SDS binding. SDS-PAGE is perhaps the most widely used method for determining the molecular weight of proteins. It is also a simple and effective method for establishing the purity of a protein, since each band on a gel corresponds to a different protein in the solution.

SDS-PAGE was carried out, as described by Laemelli (Laemmli, 1970) using a precast gel (BioRad) with a 4% stacking gel and a 10-20% gradient separating gel, to determine the purity of PPE and to check the molecular weight. A broad range pre-stained protein marker (New England BioLabs) was used as a reference to determine the molecular weight of PPE.
A 20 μl aliquot of PPE was mixed with 10 μl of loading buffer. The samples and the prestained protein marker were boiled for 5 minutes and then loaded into the wells in the gel. The gel was then placed in an electrophoresis tank containing 80 mls of running buffer. This was then poured into the tank and the gel was allowed to run for 50 minutes at 120 mA. After 50 minutes the gel was placed into a solution of 0.1% Coomassie blue R-250 in 45:45:10 % methanol:water:acetic acid. This was microwaved for 2 minutes to speed up the staining process. The gels were then destained over night using a solution of 10:10:80 % methanol:water:acetic acid. The gel was then rinsed with water and dried before analysis.

**2.1.4. Purification of PPE**

Dialysis is a convenient method of purifying proteins, particularly the removal of small molecules such as salts. Small molecules move through pores in the dialysis membrane by diffusion, when placed a less concentrated solution. Larger molecules such as proteins are too large to pass through the pores and so remain within the membrane. Proteins are usually dialysed against a buffer 1000 times the volume of the sample to be dialysed, and it takes between 16 – 24 hours for complete protein purification.

PPE was purified by dialysis to remove any buffer or salts present in the sample. Slide-a-lyzer dialysis cassettes (Pierce chemical Co) were used with a membrane of molecular weight cut off point of 3500 Da. 200 μl of PPE (150 μM or 3.89 mg/ml) was dialysed against 500 ml of 2.5 mM ammonium acetate buffer pH 8 at 4 °C for 24 hours.

**2.1.5. Determination of total PPE content- Bradford’s assay**

The Bradford assay (Bradford, 1976) is routinely used to determine the total amount of protein in a sample. When proteins bind to the Bradford reagent, which consists of an acidic solution of coomassie blue G-250, there is a shift in the A$_{max}$ from 465 nm to 595 nm. A standard curve is prepared by measuring the absorbance of various
known concentrations of bovine serum albumin (BSA) at 595 nm, after incubation with the Bradford solution. The protein concentration of a sample can then be determined from the standard curve.

A modification of the Bradford assay was used to determine the concentration of PPE. This modification involved preparing known concentrations of PPE rather than BSA, since it was found that original assay was underestimating the amount of PPE present in the sample.

2.1.5.1. Preparation of the protein reagent

50 mg of Coomassie brilliant blue-G250 was dissolved in 25 ml of 95% ethanol. To this solution 50 ml of 85% (w/v) phosphoric acid was added and this was diluted to a final volume of 500 ml.

2.1.5.2. Preparation of the standard Curve

The solutions in Table 6 were prepared and 5 mls of protein reagent was added to each standard. The contents were then mixed thoroughly and the absorbance was measured at 595 nm. The concentration of protein was then plotted against the corresponding absorbance to produce the standard curve.

Table 6: Preparation of PPE Standards for the Bradford’s Assay

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume of PPE (1 mg/ml) µl</th>
<th>Volume of H₂O (µl)</th>
<th>Concentration of PPE (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>40</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>60</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>80</td>
<td>0.2</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
2.1.5.3. Preparation of the samples

Samples of PPE were assayed in order to determine the concentration of PPE. A 100 μl aliquot of each sample was added to 5 mls of protein reagent and the contents were mixed thoroughly. The absorbance was then measured at 595 nm. The standard curve was then used to determine the concentration of PPE.

2.1.6. UV-visible spectroscopy of PPE

UV-visible spectroscopy is a useful tool to determine the effect that inhibitors have on the activity of an enzyme. A colorimetric substrate, \( N\text{-Suc-AAA-pNa} \), was used which upon hydrolysis results in the production of nitroanilines, which absorb in the visible region (410 nm) (Figure 27).

![Chemical Structures](image)

Figure 27: Hydrolysis of \( N\text{-Suc-AAA-pNa} \) by PPE.

The rate of enzyme activity can be determined by measuring the production of the nitroanilide product over a certain time period. The enzyme activity can then be compared to the rate obtained in the presence of inhibitor under the same conditions. Various kinetic parameters can be determined in this manner in particular \( k_{obs} \), \( k_{ass} \), \( t_{1/2} \), \( k_2 \) and \( K_1 \).

UV-visible spectroscopy was conducted on a Hewlett Packard 8453, equipped with a thermostated multicell transport system. The software used for the collection and
analysis of the data was HP 850 x UV-visible system. The initial rate of hydrolysis of the substrate N-Succinyl-Alanine-Alanine-Alanine-para-nitroanilide (Suc-AAA-pNa), which is catalysed by PPE (Bieth et al., 1974), was measured at 410 nm ($E_{410} = 8800 \, M^{-1} \, cm^{-1}$).

Typically 70µl of the substrate stock (100 mM) and 830µl of Tris buffer was added to a 1 ml cuvette and the reaction was initiated by the addition 100 µl of elastase (1 µM). The reaction was monitored continuously at a constant temperature of 30 °C for 300 seconds at 410 nm. A typical increase of 0.2 absorbance units was obtained.

### 2.1.6.1. Measuring the $K_m$ of Suc-AAA-pNa

The initial rate of PPE activity was measured by monitoring the absorbance at 410 nm continuously for 60 seconds at 30 °C in the presence of increasing concentrations of the substrate $N$-Suc-AAA-pNa. Typically final concentrations of 0.5, 1, 2, 4, 6 and 8 mM substrate was used. An aliquot of 900 µl of PPE buffer (50 mM tris buffer pH 8 containing 0.5 - 8 mM Suc-AAA-pNa) was added to a microcuvette. The reaction was initiated by the addition of 100 µl of elastase (1 µM). The value for the initial rate was obtain using the software package HP 850 x UV-visible system and the $K_m$ was then determined (Dixon, 1972) from the intercept on the y-axis of a plot of 1/[S] against 1/V.

### 2.1.6.2. Measuring the limits of Suc-AAA-pNa

The data from the $K_m$ experiment can be used to determine if the turnover of product by elastase is constant over the range of substrate concentration chosen which was 0.5 – 8 mM. In the $K_m$ experiment (see 2.1.6.1) the absorbance at 410 nm was measured for 60 seconds to determine the rate of activity of PPE. The absorbance at 410 nm after 60 seconds can then be plotted against the substrate concentration to determine if there is a linear appearance of product. The graph in Figure 28 shows that the appearance of product does seem to be linear.
This can be analysed further by calculating the percentage of product turnover by elastase since the absorbance at 410 nm divided by extinction coefficient of the substrate (8800 M$^{-1}$ cm$^{-1}$) is equal to the number of moles in the reaction mixture. For example in Table 7 the absorbance at 410 nm, after 1 minute’s hydrolysis of the 0.5 mM substrate, was 0.08. When divided by 8800 this equals 0.0091 mM of product produced and so elastase has converted 1.82% of the substrate into product.

Table 7: Percentage turnover of product by elastase at various concentrations of substrate.

<table>
<thead>
<tr>
<th>Initial concentration of substrate (mM)</th>
<th>Abs at 410 nM after 60 s</th>
<th>Conc of product in reaction (mM) after 60 s</th>
<th>% of product turnover by elastase (100 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.08</td>
<td>0.0091</td>
<td>1.82</td>
</tr>
<tr>
<td>1</td>
<td>0.065</td>
<td>0.0074</td>
<td>0.74</td>
</tr>
<tr>
<td>2</td>
<td>0.09</td>
<td>0.010</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>0.18</td>
<td>0.020</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>0.028</td>
<td>0.47</td>
</tr>
<tr>
<td>8</td>
<td>0.30</td>
<td>0.034</td>
<td>0.43</td>
</tr>
</tbody>
</table>
The turnover of elastase seems to be constant at about 0.5% using a final elastase concentration of 100 nM. The turnover of product is slightly higher at a substrate concentration of 0.5 mM and so perhaps this is just outside the linear range. All further experiments were carried out with a final substrate concentration of 7 mM and this is within the linear range as determined from Table 7.

2.1.6.3. Measuring the kinetic parameters of PPE inhibitors

100 μl of PPE (10 μM) was mixed with 1 μl of 3 mM of each β-lactam (Table 5) and 10 μl aliquots were taken at 0 minutes, 3 minutes, 10 minutes, 15 minutes and 20 minutes. The residual activity was then determined by adding the 10 μl aliquot to 920 μl of tris buffer and 70 μl of the substrate (100 mM). The reaction was then monitored at 410 nm and 30 °C for 300 seconds. The initial rate was then determined using the software package HP 850 x UV-visible system. E11 (Figure 26) was used as a positive control in place of the β-lactams. The initial rate of PPE in the absence of inhibitor was determined so that the residual activity of inhibited PPE could be calculated. The natural log (ln) of the residual activity (a) was then plotted against time (s) according to the equation ln a = -k_{obs}t (Kitz and Wilson, 1962) to obtain the observed rate of inactivation (k_{obs}). The apparent rate of inhibition (k_{ass}) can then be calculated from the following relationship k_{ass} = k_{obs}/[I] (Salvesen and Nagase, 1989). The time for the free enzyme concentration to decrease by 50% (t_{1/2}) can be determined since t_{1/2} = 0.693/k_{ass}[I] (Salvesen and Nagase, 1989). In order to determine the K_i, which represents the affinity of the enzyme for the inhibitor, and k_2 (the inactivation rate constant), 1/k_{obs} is plotted against 1/[I] according to the equation 1/k_{obs} = K_i/k_2[I] + 1/k_2. The intercept on the x-axis is -1/K_i and the intercept on the y-axis is 1/k_2.
2.1.7. Mass spectrometry of PPE

Electrospray Ionisation Mass Spectrometry (ESI-MS) is an extremely useful tool in the study of protein interactions. Multiply charged molecular ions are produced from a solution by spraying it under the influence of a strong electrical field. The actual mass of the protein is then determined from its mass/charge (m/z). ESI-MS is particularly useful in the study of enzyme inhibitors since it provides evidence of the formation of intermediates. The mass of an enzyme in the presence of an inhibitor is compared to a positive control. An increase in the mass of the enzyme indicates inhibition. The identification of these intermediates assists in the elucidation of the mechanism of inhibition.

2.1.7.1. Detection of covalent species by LC/MS.

Liquid chromatography is often coupled to ESI-MS to allow the removal of any buffers or salts which might interfere with ESI-MS. Reversed-phase liquid chromatography (RPLC) is useful in the separation of proteins. Hydrophobic groups are covalently attached to a hydrophilic matrix so as to provide a hydrophobic surface in which the protein absorbs. The protein can then be eluted by means of an organic gradient, most commonly acetonitrile (MeCN).

Reversed-phase liquid chromatography (RPLC) was carried out on a Waters Alliance 2790 prior to electrospray ionisation mass spectrometry (ESI-MS) to remove any buffers or unbound inhibitor. Aliquots (10 µl) were injected onto a 3 cm x 2.1 mm RP-300 aquapore octyl C₈ column and eluted using a 5 - 95 % acetonitrile gradient at 8 µl/min (Figure 29).
Figure 29: Chromatograph showing the elution of elastase after 21 minutes.

ESI-MS was carried out on a Micromass Platform II with an extended mass range to 4000 mass/charge units (m/z). A cone voltage of 70 V and a source temperature of 140 °C were employed. Samples were routinely scanned from 500 to 4000 m/z continuously over the 45 minutes chromatography run. The scan time was set to 6.5 seconds. Masslynx 3.5 was used for the collection and analysis of data. In order to obtain spectra from the TIC chromatogram, spectra were combined just above the base line for the peak. This was typically 21-22 minutes. The baseline areas at either sides of the peak (0-20 and 22-45 minutes) were subtracted to remove scans of no interest. This generated raw data such as that in (Figure 30).
Software known as Maxent was then used to deconvolute (Figure 31) this raw data. In order for maxent to process the raw data it requires an accurate peak width, the peak width of the tallest peak at 50% intensity was commonly used.
The β-lactam inhibited enzyme was subjected to LC-MS to determine if they could covalently inhibit PPE. The commercial inhibitor EI1 was used as a positive control since it is a known covalent inhibitor of PPE (Schmidt et al., 1991). Elastatinal was used as a negative control since it is a noncovalent inhibitor of elastase (Feinstein, 1976). Typically 10 μl of PPE (100 μM) in 50 mM tris buffer (pH 8.0) was incubated with 3 μl of inhibitor (1000 μM) for an hour at 30 °C prior to LC/MS. PPE in the absence of inhibitor was also subjected to LC/MS in order to determine the molecular weight before inhibition.

2.1.7.2. Monitoring time dependent inhibition

β-lactams that were found to inhibit PPE in a covalent manner were then subjected to LC/MS in order to determine if they were time dependent inhibitors. Classically 200 μl of 100 μM PPE in 50 mM tris buffer (pH 8.0) was incubated with 6 μl of inhibitor (10 mM) and 10 μl aliquots were taken at 0 minutes, 1 minutes, 5 minutes, 15 minutes, 30 minutes, 1 hour, 8 hours, 1½ days, 2 days, 5½ days, 6 days, 8 days and 10 days and were subjected to LC/MS as in 2.1.7.1.
2.2. Trypsin

2.2.1. Materials

Bovine trypsin type XIII and the colorimetric substrate BAEE (Na-Benzoyl-L-Arginine-Ethyl-Ester) was purchased from Sigma. The inhibitor controls leupeptin and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) were also purchased from Sigma. Novel β-lactams (Table 5) were synthesised by Dr James Dowden and Kevin Bailey from the University of Edinburgh and we all single diasteromers. All other reagents and chemicals were purchased from sigma unless otherwise stated.

![Figure 32: Structure of Leupeptin (N-acetyl-L-leucine-L-leucine-L-arginal).](image)

![Figure 33: Structure of 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF).](image)
2.2.2. Preparation of stock solutions and buffers

Stock solutions of trypsin (100 μM) were prepared by dissolving 0.0023g in 1 ml of ammonium acetate buffer and storing at −20 °C. The ammonium acetate buffer (2.5 mM) was prepared by dissolving 0.096 g of ammonium acetate in 500 mls of deionised water, the pH was set to 7.6. Stock solutions (10 mM) of the colorimetric substrate BAEE (Nα-Benzoyl-L-Arginine-Ethyl-Ester) were prepared by dissolving 0.017 g in 5 mls of Dimethylsulfoxide (DMSO). Stock solutions of 10 mM in DMSO were prepared for the inhibitor controls leupeptin and AEBSF and also for the β-lactam inhibitors. If more dilute concentrations were required, serial dilutions were carried out until the required concentration was reached.

2.2.3. SDS-PAGE

SDS-PAGE was used to determine the purity of trypsin and to check the molecular weight in the same manner as for PPE in section 2.1.2.

2.2.4. Determination of total trypsin content- Bradford's assay

The concentration of trypsin was also carried out by a modification of the Bradford’s assay (2.1.5). The standard curve was prepared using known concentrations of trypsin.

2.2.5. UV-visible spectroscopy of trypsin

2.2.5.1. Enzyme assay

Trypsin can catalyse the hydrolysis of N-benzoyl-arginine ethyl ester (BAEE), which results in the release of N-benzoyl-arginine and ethanol (Figure 34). This release of N-benzoyl arginine can be monitored at 253 nm (ε₂₅₃ = 808 M⁻¹ cm⁻¹).
The initial rate of hydrolysis of BAEE was monitored using a Hewlett Packard 8453 with a thermostated multicell transport system which was set to 30 °C. Typically 30 μl of the substrate stock (10 mM) and 870 μl of ammonium acetate buffer was added to a 1 ml quartz cuvette and the reaction was initiated by the addition of 100 μl of trypsin (1 μM). The reaction was monitored continuously for 300 seconds at 253 nm.

### 2.2.5.2. Measuring the $K_m$ of BAEE

The initial rate of trypsin was measured by monitoring the absorbance at 253 nm continuously for 230 seconds at 30 °C in the presence of increasing concentrations of substrate. Typically substrate concentrations of 5, 10, 50, 100, 300 and 500 μM were used. An aliquot of 900μl of buffer (67 mM sodium phosphate buffer pH 7.6 containing 5 - 500 μM BAEE) was added to a quartz cuvette. The reaction was initiated by the addition of 100 μl of trypsin (1 μM) and was monitored continuously at a constant temperature of 30°C for 300 seconds at 253 nm. The value for the initial rate was obtained using the software package HP 850 x UV-visible system and the $K_m$ was determined (Dixon, 1972) from the intercept on the y-axis of a plot of $1/[S]$ against $1/V$.

### 2.2.5.3. Measuring the limits of BAEE

The data from the $K_m$ experiment was also used to determine if the turnover of product by trypsin is constant over the range of substrate concentration chosen which was 5 - 500 μM. In the $K_m$ experiment (see 2.2.5.2) the absorbance at 253 nm was measured for 300 seconds to determine the rate of activity of trypsin. The absorbance at 253 nm after 300 seconds can then be plotted against the substrate concentration.
to determine if there is a linear appearance of product. The graph in Figure 35 shows that the appearance of product does seem to be linear.

![Graph showing the increase in absorbance at 253 nm with increasing substrate concentration.](image)

Figure 35: Graph showing the increase in absorbance at 253 nm with increasing substrate concentration.

The percentage of product turnover by trypsin was calculated since the absorbance at 253 nm divided by extinction coefficient of the substrate (808 M\(^{-1}\) cm\(^{-1}\)) is equal to the number of moles in the reaction mixture.
The turnover of trypsin does not seem to be as constant as it was for PPE since % turnover varies from 99% to 52%. The turnover is also much higher averaging at about 75% for trypsin (100 nM). All further experiments were carried out with a substrate concentration of 0.3 mM.

### Measuring the kinetic parameters of trypsin inhibitors

100 μl of trypsin (10 μM) was mixed with 1 μl of each β-lactam (3 mM) and 10 μl aliquots were then taken at 0, 10, 20, 30, 60 and 180 minutes. The residual activity was then determined by adding the aliquot to 960 μl of buffer and 30 μl of substrate (10 mM). The reaction was then monitored at 253 nm and 30 °C for 300 seconds in a 1 ml quartz cuvette. The kinetic parameters were determined as in section 2.1.6.3. This was then repeated using a final inhibitor concentration of 1 and 5 μM.

### Mass spectrometry of trypsin

#### Detection of covalent species by LC/MS

The β-lactams were subjected to LC/MS, in order to determine whether the β-lactams were covalent inhibitors of trypsin. Typically 10 μl of trypsin (100μM) was incubated with 3 μl of inhibitor (1000 μM) for 24 hours prior to LC/MS. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was used a positive control since it was known to covalently inhibit trypsin, leupeptin was used as a
negative control since it is a known reversible inhibitor of trypsin (Kuramochi et al., 1979). Trypsin was also analysed in the absence of inhibitor to determine the molecular weight before inhibition.

ESI-MS was carried out on a Micromass Platform II with an extended mass range to 4000 mass/charge units (m/z). A cone voltage of 70 V and a source temperature of 140 °C were employed. Samples were routinely scanned from 500 to 4000 m/z continuously over the 45 minutes chromatography run. The scan time was set to 6.5 seconds.

![Chromatograph showing the elution of trypsin after 14 minutes.](image)

Masslynx 3.5 was used for the collection and analysis of data. In order to obtain spectra from the chromatogram (Figure 36), spectra were combined just above the base line for the peak. This was typically between 14 and 16 minutes. The baseline areas at either sides of the peak (0-13 and 17-45 minutes) were subtracted to remove scans of no interest. This generated raw data such as that in (Figure 37).
Maxent was also used to deconvolute the trypsin spectra to obtain the mass of trypsin (Figure 38). In order for maxent to obtain the deconvoluted spectra it needs to know the peak width of the sample. Typically the peak width of the tallest peak at 50% intensity was used.
2.2.6.2. Monitoring time dependent inhibition

The β-lactams which were found to covalently bind to trypsin were subjected to LC-MS in order to determine whether or not inhibition is time dependent. Typically 200 µl of 100 µM trypsin was incubated with 6 µl of inhibitor (10 mM) and 10 µl aliquots were then taken at 0 minutes, 1 minute, 30 minutes, 1 hour, 30 hours, 3 days and 7 days and subjected to LC/MS as in section 2.2.6.1.

2.2.6.3. Detection of non-covalent species

ESI-MS can also be used to detect non-covalent species. Preserving enzyme activity is of key importance in this case, therefore the organic solvents used during LC/MS cannot be used. Instead the protein can be directly infused into instrument and low source temperatures and cone voltages are required to prevent protein unfolding.

The β-lactam compounds were directly infused into the electrospray ionisation mass spectrometer to determine if they could inhibit trypsin using non-covalent interactions. Samples were directly infused into the instrument using a Harvard syringe pump and a flow rate of 3 µl/min. The cone voltage was set to 40V and a source temperature of 40°C was employed. Samples were scanned from 1000 to 4000 m/z. Typically 10 µl of trypsin (100µM) was incubated with 3 µl of inhibitor (1000 µM) for 24 hours prior to LC/MS. Trypsin in the absence of inhibitor was used as negative control and leupeptin was used as a positive control since it is a known reversible inhibitor of trypsin.
2.3. Errors

Five samples of PPE were prepared and the rate of hydrolysis of \( N\text{-Suc-AAA-pNa} \) was determined as in 2.1.6. The average rate and the standard deviation were calculated (Table 9). The standard deviation was then converted to a percentage, which was found to be approximately 36%. This served as a guide to determine the overall errors involved in obtaining a UV-visible spectroscopy measurement and included the errors involved in weighing and preparing both the samples and the buffers solutions which the samples are run in, and the errors from the UV-visible spectrophotometer as well.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Rate of hydrolysis of ( N\text{-Suc-AAA-pNa} ) by PPE (AU/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( 2.764 \times 10^{-4} )</td>
</tr>
<tr>
<td>2</td>
<td>( 5.255 \times 10^{-4} )</td>
</tr>
<tr>
<td>3</td>
<td>( 2.736 \times 10^{-4} )</td>
</tr>
<tr>
<td>4</td>
<td>( 4.615 \times 10^{-4} )</td>
</tr>
<tr>
<td>5</td>
<td>( 4.358 \times 10^{-4} )</td>
</tr>
<tr>
<td>Average ± Standard deviation</td>
<td>( 3.94 \times 10^{-4} ± 1 \times 10^{-4} ) (36 %)</td>
</tr>
</tbody>
</table>

In the same manner five samples of PPE were prepared and the mass was determined by ESI-MS to obtain a guide for the overall errors involved in obtaining a mass spectrometry reading (Table 10). This error was found to be about 5 mass units therefore all MS data was rounded up to the nearest unit. The standard deviation was again converted to a percentage, which was calculated to be 0.02%, this indicates that the errors in using the mass spectrometer is less than the errors involved in using the UV-visible spectrometer.
Table 10: Determination of errors for mass spectrometry.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Mass of PPE (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25904</td>
</tr>
<tr>
<td>2</td>
<td>25892</td>
</tr>
<tr>
<td>3</td>
<td>25902</td>
</tr>
<tr>
<td>4</td>
<td>25900</td>
</tr>
<tr>
<td>5</td>
<td>25907</td>
</tr>
<tr>
<td>Average ± standard deviation</td>
<td>25903 ± 5 (0.02 %)</td>
</tr>
</tbody>
</table>

*Theoretical $M_r = 25898.06$

Where multiple identical experiments were carried out, the standard deviations were calculated. Although the calculation of errors is an important part of any scientific documentation the main aim of this work was determine any general trends in the data rather than acquire complete accuracy.
3. Results & discussion: Elastase

3.1. SDS-PAGE

The samples of elastase were analysed by SDS-PAGE to ensure that the level of purity remained constant between different batches. SDS-PAGE also confirmed the claim from the supplier (Calbiochem) that the enzyme was more than 98% pure (Figure 39), since only one band was visible after staining.

![Figure 39: SDS-PAGE of elastase](image)

3.2. Total PPE content

The Bradford assay (Bradford, 1976) is the most common method for determining the total protein concentration in a sample. The assay is based on the knowledge that when proteins bind to coomassie blue G-250 a shift the $A_{\text{max}}$ from 465 to 595 nm occurs. Preparation of a standard curve then allows the concentration of protein to be determined.

It was found that the BSA standards, normally used to prepare the standard curve, were underestimating the concentration of protein in the sample as can be seen from...
Figure 40. A sample which was found to have an absorbance at 595 nm of 0.2 would have 0.30 mg/ml of protein as determined from the BSA standard curve, but the same protein sample would have 0.84 mg/ml protein in it as determined from the PPE standard curve.

This almost 3 fold difference is due to fact that BSA has more aromatic amino acids than PPE. The coomassie blue in the Bradford’s reagent binds to aromatic amino acids in proteins and since BSA contains more aromatic groups than PPE, the same concentration would give a greater absorbance reading. BSA standards were replaced with PPE standards to alleviate this problem.
3.3. Determination of inhibition constants for PPE

3.3.1. Kinetic parameters of PPE inhibitors

The β-lactams (Table 11) were tested as inhibitors of PPE using UV-visible spectroscopy. The commercial inhibitor Eli was used as a positive control. The log of residual activity was plotted against time and the observed rate of inactivation \((k_{obs})\) was obtained from the slope of the line (2.1.6.3). A typical graph is shown in Figure 41. The errors on the graph are likely to be quite large since only few points were used we can still determine the general trend of the graph.

![Figure 41: Plot of residual activity against time for the inhibition of PPE by Eli](image-url)
Table 11: Structures of compounds tested for inhibition of PPE

<table>
<thead>
<tr>
<th>Name Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td><img src="image" alt="Structure of JD260" /></td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image" alt="Structure of JD261" /></td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image" alt="Structure of JD263" /></td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image" alt="Structure of JD264" /></td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image" alt="Structure of JD417" /></td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image" alt="Structure of JD420" /></td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image" alt="Structure of JDx3" /></td>
</tr>
<tr>
<td>E11</td>
<td><img src="image" alt="Structure of E11" /></td>
</tr>
</tbody>
</table>
The values for the apparent rate of inhibition ($k_{\text{ass}}$) (Figure 42) were calculated from $k_{\text{obs}}/I$. All were found to be effective PPE inhibitors with the most potent ones being JD260 ($k_{\text{ass}} = 9875 \pm 2474 \text{ M}^{-1} \text{ s}^{-1}$), JDx3 ($k_{\text{ass}} = 8799 \pm 1511 \text{ M}^{-1} \text{ s}^{-1}$) and JD261 ($k_{\text{ass}} = 6032 \pm 112 \text{ M}^{-1} \text{ s}^{-1}$). JD417, JD264 and JD263 were not quite as potent and JD420 was found to be the least potent $\beta$-lactam which still had a $k_{\text{ass}} = 1143 \pm 101 \text{ M}^{-1} \text{ s}^{-1}$. The commercial inhibitor Eli was not found to be particularly potent with a $k_{\text{ass}}$ comparable to JD263. The errors for the $k_{\text{ass}}$ values are all extremely variable since JD261 and JD420 both have quite low errors while, Eli, JDx3, JD260, JD263 and JD264 all have larger errors and JD417 has a much larger error. This is a little unusual since all the $\beta$-lactams were analysed using identical conditions and so the errors should have been constant.

![Figure 42: $k_{\text{ass}}$ of PPE Inhibitors. Error bars show the standard deviation.](image)

The half-lives ($t_{1/2}$) for inactivation by the $\beta$-lactams were also determined and are shown in Table 12. Again JD260 and JDx3 were most effective since these $\beta$-lactams were faster at inhibition with $t_{1/2}$ of 3 and 4 minutes respectively at concentrations of 0.3 $\mu$M. JD420 was also the slowest inhibitor since a concentration of 0.3 $\mu$M took about 36 minutes to inactivate PPE by 50%.
Table 12: Half-lives (t½) of PPE inhibitors in minutes

<table>
<thead>
<tr>
<th>[Inhibitor] (µM)</th>
<th>JD260</th>
<th>JD261</th>
<th>JD263</th>
<th>JD264</th>
<th>JD417</th>
<th>JD420</th>
<th>JDx3</th>
<th>EI1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4</td>
<td>20</td>
<td>35</td>
<td>21</td>
<td>89</td>
<td>95</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>0.3</td>
<td>3</td>
<td>6</td>
<td>21</td>
<td>12</td>
<td>4</td>
<td>36</td>
<td>4</td>
<td>36</td>
</tr>
</tbody>
</table>

$k_{obs}$ was replotted against [I] (Figure 43) and the data was fitted to the curve $k_{obs} = k_2[I]/(K_i + [I])$ in order to obtain values for the inhibition constant ($K_i$), the turnover number ($k_2$) and the specificity constant ($k_2/K_i$) (Table 13).

Figure 43: Plot of $k_{obs}$ versus [I] after inhibition of PPE with β-lactams

None of the inhibitors was found to be exceptional tight binding, although with $K_i$'s in the millimolar range they are still effective enzyme inhibitors. The $k_2/K_i$ values range from $1\times10^3$ M to almost $1\times10^4$ M confirming that these are indeed effective elastase inhibitors although more potent ones have been reported (Bernstein et al., 1994) and (Edwards and Bernstein, 1994). Yet again the values show that JD260, JDx3 and JD417 are the best inhibitors and JD420 is the least potent. The commercial inhibitor EI1 has similar values to JD263 and so was not one of the best inhibitors.
Table 13: k₂ and Kᵢ of PPE Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>k₂ (sec⁻¹)</th>
<th>Kᵢ (mM)</th>
<th>k₂/Kᵢ (M⁻¹ sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td>7</td>
<td>0.8</td>
<td>9468</td>
</tr>
<tr>
<td>JD261</td>
<td>13</td>
<td>2.1</td>
<td>6229</td>
</tr>
<tr>
<td>JD263</td>
<td>6</td>
<td>3.3</td>
<td>1951</td>
</tr>
<tr>
<td>JD264</td>
<td>9</td>
<td>2.5</td>
<td>3460</td>
</tr>
<tr>
<td>JD417</td>
<td>15</td>
<td>1.8</td>
<td>8111</td>
</tr>
<tr>
<td>JD420</td>
<td>10</td>
<td>9.2</td>
<td>1086</td>
</tr>
<tr>
<td>JDx3</td>
<td>11</td>
<td>1.2</td>
<td>9417</td>
</tr>
<tr>
<td>Eli</td>
<td>6</td>
<td>3.2</td>
<td>1875</td>
</tr>
</tbody>
</table>

3.3.1.1. Comparison of inhibitor efficiency as evidenced by UV

Analysis of all the data consistently shows the best β-lactam inhibitors are JD260, JDx3 and JD417. These compounds had the highest affinity for the enzyme (Kᵢ) and JD417 and JDx3 also had the highest rate of inactivation (k₂). Therefore they quickly inactivated the enzyme and bound tightly.

The β-lactams JD261 and JD264 are not quite as effective, they were slower at inhibition because they had lower rates of association (kₐss) and longer half-lives (t½). These compounds also had a lower value for k₂/Kᵢ.

The compounds that were least effective were JD263 and JD420. These had the lowest affinity for the enzyme (Kᵢ). They were also slowest at inhibition since they had low rates of inactivation (k₂) and association (kₐss) as well as high t½ values. The commercial inhibitor Eli, which was used as a control, behaved very similar to JD263 and so most of our β-lactams were more potent than Eli.
3.4. ESI-MS of PPE-Inhibitor complexes

Electrospray Ionisation Mass Spectrometry (ESI-MS) was used to study the interaction of the β-lactams with porcine pancreatic elastase (PPE). ESI-MS is particularly useful in the study of proteins since it produces sample ions, which carry multiple charges. This means that compounds with high molecular weights can also be measured.

Mass spectrometry is also a useful tool in the study of enzyme inhibition since intermediates can be detected which aids the identification of the inhibition mechanism.

3.4.1. Mechanism of elastase inhibition by β-lactams

The classic mechanism of elastase inhibition by β-lactams (see Figure 46) is based on the work by the Merck group (Underwood et al., 1995). The Merck group used electrospray ionisation mass spectrometry (ESI-MS) and two-dimensional NMR techniques to identify β-lactam-derived E-I complexes. Human leukocyte elastase (HLE) was incubated with a β-lactam known as L-680,833 (Figure 44) and analysed by ESI-MS. The ESI-MS spectrum showed an increase of 333 Da over the mass of the free enzyme. This mass increase was found to be consistent with the formation of a carbinolamine. Analysis of HLE and PPE with related compounds also identified inhibitor complexes that corresponded to the formation of a carbinolamine.

![Figure 44: Structure of L-680,831 (β-lactam derivative)](image-url)
The mechanism (Figure 46) (mechanism 1) for the formation of this carbinolamine involves the hydroxyl group of serine_{195} undergoing nucleophilic addition to the lactam carbonyl. Formation of a tetrahedral intermediate occurs, which then collapses to cause the subsequent formation of an acyl-enzyme, resulting in opening of the β-lactam ring. The leaving group in the C4 position is then eliminated to form an imine. Histidine_{57} picks up a proton from the group at the C3 position of the β-lactam and water then adds at the C4 position forming a carbinolamine. The carbinolamine can then break down to form various aldehyde compounds.

Previous X-ray crystallography work on the β-lactam compound JD261 (Taylor et al., 1999) identified the enzyme-inhibitor complex of this compound in the active site of PPE. The E-I complex (Figure 45) was consistent with the formation of a new acyl-enzyme intermediate. The active site histidine does not make any hydrogen bonds directly with JD261, instead it forms a hydrogen bond with a nearby water molecule that is well positioned to carry out nucleophilic attack. The E-I complex has no traces of substituents on the 3 and 4 positions of the β-lactams and so does not fit with any of the previously known mechanisms of β-lactam inhibition.

![Figure 45: A difference electron density map showing the product of JD261 complexed with PPE. Taken from Taylor et al., 1999.](image-url)
Taylor et al. (1999), also showed that this JD261 inhibited elastase via a different mechanism to the one identified by Merck (mechanism 1) which we will label as mechanism 2. It is proposed that the mechanism of reaction (Figure 46) proceeds similar to mechanism 1 in that acylation of active site serine occurs with loss of the substituent on the C4 of the β-lactam ring and subsequent formation of the imine. Thereafter instead of water being added at C4 position, the hydroxyethyl substituent on C3 provides an alternative pathway for reaction by a retro-aldol reaction to generate the acyl-enzyme intermediate. The identity of the acyl-enzyme complex was also verified by electrospray ionisation mass spectrometry and the mass of the covalent species corresponds to the species observed by X-ray crystallography. It was also discovered that the other members of the series (JD260, JD263 and JD264) behaved in the same manner.

The study on the first round of β-lactam inhibitors (JD260-264), which varied the substituents at the R1 position (Figure 14), then led to the design and synthesis of a second round of β-lactam inhibitors, namely JD417, JD420, JDx1 and JDx3 (Table 5). These second round of inhibitors had the same R1 group but varied their substituents at the C3 position.

It was expected that since JD417 has exactly the same C3 group as the first series of β-lactams, that its mechanism of action should be the same, producing an acyl-enzyme intermediate via mechanism 2 (Figure 46). JDx1 has a C3 group similar to the first series of β-lactams since it also has a hydroxyl group, but it also possesses an extra methyl group. The presence of the hydroxyl group means that JDx1 should also inhibit PPE via mechanism 2. JD420 and JDx3 do not contain hydroxyl groups at the C3 position and so their mechanism of inhibition should be similar to mechanism 1. Expected inhibitor fragments are shown in Table 14.
Figure 46: In mechanism 1 the active site serine of elastase is acylated by the β-lactam ring to generate a tetrahedral intermediate. This collapses to cause the formation of an acyl-enzyme, the β-lactam ring opens and the substituents on the C4 group are eliminated to form an imine. Water then adds at the C4 position forming a carbinolamine. The carbinolamine then breaks down to generate aldehydes. In mechanism 2 the presence of a hydroxyl group on the C3 substituent leads to a retro-aldol reaction that forms a much more stable intermediate. The intermediates shown in the blue boxes have been visualised by ESI-MS.
Table 14: predicted structures and MW's of fragments resulting from inhibition of PPE by novel β-lactams

<table>
<thead>
<tr>
<th>Name</th>
<th>β-lactam</th>
<th>acyl-enzyme intermediate</th>
<th>carbinolamine</th>
<th>aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td><img src="image1.png" alt="Structure" /></td>
<td><img src="image2.png" alt="Structure" /></td>
<td><img src="image3.png" alt="Structure" /></td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image5.png" alt="Structure" /></td>
<td><img src="image6.png" alt="Structure" /></td>
<td><img src="image7.png" alt="Structure" /></td>
<td><img src="image8.png" alt="Structure" /></td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image9.png" alt="Structure" /></td>
<td><img src="image10.png" alt="Structure" /></td>
<td><img src="image11.png" alt="Structure" /></td>
<td><img src="image12.png" alt="Structure" /></td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image13.png" alt="Structure" /></td>
<td><img src="image14.png" alt="Structure" /></td>
<td><img src="image15.png" alt="Structure" /></td>
<td><img src="image16.png" alt="Structure" /></td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image17.png" alt="Structure" /></td>
<td><img src="image18.png" alt="Structure" /></td>
<td><img src="image19.png" alt="Structure" /></td>
<td><img src="image20.png" alt="Structure" /></td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image21.png" alt="Structure" /></td>
<td><img src="image22.png" alt="Structure" /></td>
<td><img src="image23.png" alt="Structure" /></td>
<td><img src="image24.png" alt="Structure" /></td>
</tr>
<tr>
<td>JDx1</td>
<td><img src="image25.png" alt="Structure" /></td>
<td><img src="image26.png" alt="Structure" /></td>
<td><img src="image27.png" alt="Structure" /></td>
<td><img src="image28.png" alt="Structure" /></td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image29.png" alt="Structure" /></td>
<td><img src="image30.png" alt="Structure" /></td>
<td><img src="image31.png" alt="Structure" /></td>
<td><img src="image32.png" alt="Structure" /></td>
</tr>
</tbody>
</table>

*N/A = Not available*
3.4.2. Detection of covalent inhibition by β-lactams

LC/MS was used to detect any covalent inhibition by the β-lactams and confirm their mechanism of action. The β-lactams (Table 5) underwent reversed-phase liquid chromatography (RPLC) immediately before entering the mass detector to remove any salts, buffer or unreacted inhibitor. A commercial inhibitor (El1) that covalently inhibits PPE was used as a positive control. El1 belongs to a family of compounds known as O-acyl-hydroxymates, which are thought to inhibit serine proteases using a mechanism involving the Lossen rearrangement (Groutas et al., 1986) (Figure 47).

![Diagram of El1 inhibition of PPE](image)

Figure 47: Mechanism of El1 inhibition of PPE

El1 was found to covalently inhibit PPE since a peak was detected at 26222 (Figure 48), which represents native PPE plus 329 Da. The expected size of the fragment formed after PPE inhibition by El1 is 329 Da and so the data correlates well with our knowledge of how the inhibitor should interact with the enzyme. To our knowledge this is the first reported case whereby El1 has been analysed by ESI-MS.
The β-lactams also behaved as expected. JD260 (Figure 49), JD261 (Figure 50), JD263 (Figure 51), JD264 (Figure 52), and JD417 (Figure 53) which all have a hydroxyl group present at the C3 position, were shown to react via mechanism 2 (Figure 46) as the corresponding covalent acyl-enzyme intermediates were observed (Table 15) which correlated well the size of fragment they were expected to generate (Table 14). A peak corresponding to the carbinolamine was also occasionally observed with these compounds but it was always much smaller than the peak corresponding to the acyl-enzyme intermediate. The observation that the acyl-enzyme intermediate peak is always predominant suggests that although these compounds are free to react by both mechanisms, mechanism 2 is perhaps kinetically favoured.

JD420 (Figure 54) and JDx3 (Figure 62), both of which lack a hydroxyl group present at the C3 position, were found to react via mechanism I (Figure 46) since fragments were observed (Table 15) which closely resemble the expected fragment (Table 14) for the formation of the carbinolamine.

JDx1 was an intermediate used in the synthesis of another β-lactam and so is slightly different from the others in the series. It has an extra methyl group at the C3 position and so it was interesting to find out if this larger C3 group would fit into the active site of PPE. It was found that only the native enzyme could be detected (Figure 55) and so there was no evidence that JDx1 inhibits PPE at all. Therefore JDx1 was the only member of the β-lactams, which did not react as expected. It seems that perhaps the active site of PPE cannot accommodate the larger C3 group of JDx1 and so catalysis cannot occur.
Figure 48: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of El1 for 1 hour. The peak at 25893.14 represents the native enzyme, the peak at 26222.04 represents the inhibitor fragment.
Figure 49: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD260 for 1 minute. The peak at 25894.47 represents the native enzyme, the major peak at 26081.88 represents the stable acyl-enzyme intermediate and the peak at 26141.18 represents the carbinolamine.
Figure 50: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD261 for 5 minutes. The peak at 25893.42 represents the native enzyme and the peak at 26130.80 represents the stable acyl-enzyme intermediate.
Figure 51: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD263 for 20 minutes. The peak at 25891.09 represents the native enzyme and the peak at 26045.71 represents the stable acyl-enzyme intermediate.
Figure 52: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD264 for 1 minute. The peak at 25893.18 represents the native enzyme, the major peak at 26095.04 represents the stable acyl-enzyme intermediate and the peak at 26154.78 represent the carbinolamine.
Figure 53: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD417 for 15 minutes. The peak at 25893.69 represents the native enzyme and the peak at 26083.47 represents the stable acyl-enzyme intermediate.
Figure 54: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JD420 for 1 hour. The peak at 25905.57 represents the native enzyme and the peak at 26121.94 represents the carbinoyl amine.
Figure 55: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JDx1 for 1 hour. The peak at 25899.79 represents the native enzyme.
Figure 56: Deconvoluted electrospray ionisation mass spectrum of PPE incubated with a 3 fold molar excess of JDx3 for 1 minute. The peak at 25899.58 represents the native enzyme and the peak at 26132.00 represents the carbinoyl amine.
Table 15: Size of species resulting from inhibition of PPE by $\beta$-lactams as determined by mass spectrometry. Errors are standard deviations.

<table>
<thead>
<tr>
<th>Name</th>
<th>$\beta$-lactam</th>
<th>acyl-enzyme intermediate</th>
<th>carbinolamine</th>
<th>aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Structure</td>
<td>Mass (Da)</td>
<td>Observed Mass (Da)</td>
<td>Expected Mass (Da)</td>
</tr>
<tr>
<td>JD260</td>
<td><img src="image" alt="Structure" /></td>
<td>483</td>
<td>190 ± 9</td>
<td>189</td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image" alt="Structure" /></td>
<td>533</td>
<td>233 ± 9</td>
<td>239</td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image" alt="Structure" /></td>
<td>449</td>
<td>151 ± 4</td>
<td>155</td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image" alt="Structure" /></td>
<td>497</td>
<td>199 ± 7</td>
<td>203</td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image" alt="Structure" /></td>
<td>410</td>
<td>187 ± 6</td>
<td>189</td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image" alt="Structure" /></td>
<td>392</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image" alt="Structure" /></td>
<td>394</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* N/A = Not available
3.4.2.1. **Comparison of inhibitor mechanisms**

LC/MS confirms that the first series of β-lactam inhibitors (JD260-264) inhibited PPE via a novel mechanism (mechanism 2, Figure 46). The β-lactams all had a hydroxyl group at the C3 position that allowed a retro-aldol reaction to take place which resulted in the formation of a stable acyl-enzyme intermediate. JD417 from the second series of β-lactam inhibitors also reacted via mechanism 2 since it has exactly the same C3 group as the first series.

JDx1 has a similar C3 group, it has the hydroxyl group but it also contains an extra methyl group. It was thought that since it had the hydroxyl group it would react via mechanism 2 like the others. This was not the case since no inhibition was observed. It is likely that the extra methyl group makes it too large to enter the active site of elastase.

JD420 and JDx3 do not contain a hydroxyl group and as expected they were found to inhibit PPE via mechanism 1 (Figure 46) generating carbinolamines.

3.4.3. **Time dependent inhibition of β-lactams**

It was decided to investigate the time dependent inhibition of elastase by the novel β-lactams using electrospray ionisation mass spectrometry. Time dependent inhibition of PPE by several types of irreversible inhibitors has been reported by Aplin *et al.*, (Aplin *et al.*, 1992, Aplin *et al.*, 1993). Aplin found that the intensity of the peaks corresponding to the inhibited enzyme increased with incubation time. When the incubation time was increased to 24 hours reactivation of the peak corresponding to the active enzyme was also observed.

This reactivation is thought to occur due to hydrolysis of the acyl-enzyme intermediate. Green *et al.*, (Green *et al.*, 1995) propose several possible mechanisms for the reactivation of PPE after inhibition by various β-lactams.
The β-lactams that inhibited elastase via covalent attachment to the enzyme in 3.4.2 were incubated with PPE. Aliquots of the PPE/inhibitor mixture were removed at certain time intervals ranging from 1 minute to 10 days and LC/MS was carried out. The peak height of native PPE at each time point was measured, compared to the peak height of inhibited PPE and expressed as a percentage of activity (Figure 57). Graphs of % activity against time were then plotted in order to determine whether the inhibitors were reacting in a time dependent manner and to compare the general trends of the graphs. The half-lives ($t_{1/2}$) and rate of association ($k_{ass}$) for each of the β-lactams were also calculated in the same manner as for 2.1.6.3. Calculation of peak heights in this way is acceptable only if the peak widths of the samples that are being compared are nearly the same width. If different samples have variable peak widths then a more accurate method of comparison would be to measure the area under each peak and compare peak areas instead.

The time dependence study showed that the β-lactams reacted in a time dependent manner. Figure 58 shows how the intensity of the peaks change over time as PPE is inhibited by JD417. The time dependence graphs (Figure 59 -Figure 65) show a general trend whereby the activity of PPE is reduced over time, in some cases to 0%. The activity of PPE then increases as the inhibitor is reactivated. These graphs give valuable information on which β-lactams are fast inhibitors, which are more stable and so inhibit for longer periods of time and which are less stable and so are quickly reactivated.
Figure 57: Example of the calculation of % activity from the LC/MS spectra of PPE after incubation with β-lactams. The example shows PPE after incubation with a 3 fold molar excess of JD260 for 52 hours.
Figure 58: Time dependent inhibition of PPE by JD417 after incubation for 15 minutes, 24 hours and 96 hours.
JD260 (Figure 59) was one of the fastest of the β-lactams to inhibit PPE, since it had a $t_{1/2}$ of about 30 seconds. After 5 minutes all of the native enzyme had become inhibited. The inhibitor is also relatively stable since it only starts to reactivate after about 24 hours and it takes 10 days before the enzyme is completely reactivated.

JD261 (Figure 60) was slightly slower at inhibition with a $t_{1/2}$ of about 5 minutes. It was also less stable than JD260 since it starts to reactivate between 1 and 8 hours, although it still takes about 10 days before reactivation is complete. Complete inhibition of PPE by JD261 was not observed, the enzyme seemed to reactivate before 0% activity was reached. The lowest activity of PPE observed was 16% after 30 minutes of incubation with JD261.

JD263 (Figure 61) was the slowest of the β-lactams to inhibit PPE. It had a $t_{1/2}$ of more than 10 minutes. The enzyme started to reactivate before 30 minutes, and after only 31 hours the enzyme had regained 91% of its activity. JD263 also seemed to reactivate before complete inhibition was observed. The enzyme reached 40% residual activity after 15 minutes incubation but soon reactivated since at 30 minutes incubation it reached 70% activity. This suggests that JD263 is less stable than JD261.

JD264 (Figure 62) was much faster at inhibition with $t_{1/2}$ of about 2 minutes. After 15 minutes no trace of the enzyme could be detected. Although reactivation started quite quickly (between 1 and 8 hours) by 9 days only 28% of the enzyme had reactivated. Therefore JD264 was fairly stable, more so than JD261 and JD263.
JD417 (Figure 63) was also fast at inhibition since it had a $t_{1/2}$ of about 2 minutes and by 20 minutes all of the enzyme was inhibited. The inhibitor was quite stable since reactivation started at about 1 hour and took 8 days to complete which was similar to JD260, JD261 and JD264.

JD420 (Figure 64) was quite slow at inhibition, with a $t_{1/2}$ of 10 minutes it inhibits in a similar time scale to JD263. It is also not particularly stable since reactivation starts at less than an hour and is almost complete by 31 hours.

JD$x$ (Figure 65) is the most reactive of the β-lactams towards inhibition of PPE. It has a $t_{1/2}$ of less than 30 seconds and it takes less than 3 minutes for the enzyme to become completely inactivated. Although it is one of the fastest inhibitors it is also the least stable. Reactivation starts at after 15 minutes and it takes less than hour for PPE to regain its activity.

![Graph showing time dependence inhibition of JD260 on PPE.](image)
Figure 60: Graph showing time dependence inhibition of JD261 on PPE.

Figure 61: Graph showing time dependence inhibition of JD263 on PPE.

Figure 62: Graph showing time dependence inhibition of JD264 on PPE.
Figure 63: Graph showing time dependence inhibition of JD417 on PPE.

Figure 64: Graph showing time dependence inhibition of JD420 on PPE.

Figure 65: Graph showing time dependence inhibition of JDx3 on PPE.
3.4.3.1. Comparison of time dependent inhibition by β-lactams

As can be seen from Table 16 each of the compounds significantly inactivate PPE (less than 50% residual activity) for various lengths of time. With the exception of JD263 all of the compounds that inhibit PPE via mechanism 2 (Figure 46), such as JD260-264 and JD417, reactivate at a much slower rate than those that inhibit via mechanism 1, like JDx3 and JD420.

The β-lactams which inhibit via mechanism 2, cause a reduction in the activity of PPE by more than 50% for several hours (48 - 218 hrs) whereas JD420 and JDx3 only cause 50% inhibition for between 30 and 45 minutes. This is about 300-fold difference, which is quite significant.

Even more interesting is the observation that the JD260-264 series (except JD263) all inhibit for about 218 hrs, which is almost 5 times longer than JD417, which only does so for 48 hrs. If we compare JD417 with JD260 the only structural difference between them is the group at the C4 position. When designing these compounds it was thought that differences at the C4 position would have very little impact on the kinetics of these compounds since upon β-lactam catalysis the C4 group is quickly eliminated. This data suggests that perhaps this group is more important than originally anticipated. It is possible that structural differences at the C4 position forces the compound to bind to different pockets in the active site, which have an effect on the stability of the inhibitor.
Table 16: $k_{ass}$ and <50% PPE activity of β-lactams as evidenced by ESI-MS.

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>$k_{ass}$ (M$^{-1}$ S$^{-1}$)</th>
<th>Length time in which PPE &lt;50% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td>506 ± 139</td>
<td>1 min – 218 hrs (218 hrs)</td>
</tr>
<tr>
<td>JD261</td>
<td>149 ± 197</td>
<td>5 min – 218 hrs (218 hrs)</td>
</tr>
<tr>
<td>JD263</td>
<td>15 ± 19</td>
<td>15 min – 30 min (0.25 hrs)</td>
</tr>
<tr>
<td>JD264</td>
<td>155 ± 187</td>
<td>1 min – 218 hrs (218 hrs)</td>
</tr>
<tr>
<td>JD417</td>
<td>62 ± 78</td>
<td>3 min – 48 hrs (48 hrs)</td>
</tr>
<tr>
<td>JD420</td>
<td>0.3</td>
<td>15 min – 60 min (0.75 hrs)</td>
</tr>
<tr>
<td>JDx3</td>
<td>1194 ± 2049</td>
<td>1 min – 30 min (0.5 hrs)</td>
</tr>
</tbody>
</table>

3.4.4. Comparison of β-lactam inhibition as evidenced by MS

JD260 is probably the most potent of the β-lactams. It has a fast half-life ($t_{1/2}$), a high rate of association ($k_{ass}$) and one of the slowest rates of reactivation, therefore it is a fast and stable inhibitor of PPE. JDx3 is also one of the most potent β-lactams with a fast $t_{1/2}$ and a high $k_{ass}$, this means that it is a fast inhibitor but it also has a fast rate of reactivation and so doesn’t inhibit for very long. JD264, JD417 and JD261 are not as potent since they have slower $t_{1/2}$ and lower values for $k_{ass}$ but they all have slow rates of reactivation and so are quite stable. JD420 and JD263 are the least potent inhibitors, they have the lowest values of $k_{ass}$ and the slowest $t_{1/2}$ therefore they are not fast inhibitors and since they have the fast reactivation rates they are not very stable. JDx1 showed no evidence of inhibition.
4. Results and Discussion: Trypsin

4.1. SDS-PAGE

Analysis by SDS-PAGE indicated that the trypsin obtained from Sigma was relatively pure since although there were 4 bands visible (Figure 66), the trypsin band (MW ~23000) was clearly more concentrated than the others. Since trypsin is a protease, which has the ability to degrade itself, the minor bands may be due to trypsin autolysis. One way to determine if these lower molecular weight bands are degraded trypsin is to leave trypsin at room temperature and analyse aliquots by SDS-PAGE over time. If the bands are caused by trypsin autolysis then the higher molecular weight bands should become less concentrated whereas the lower molecular weight bands should become more concentrated.

![Figure 66: SDS-PAGE of trypsin](image)

4.2. Total Trypsin content

The Bradford assay was also used to determine the amount of protein in trypsin. As in 2.1.5 the BSA standards (Figure 67) in the Bradford’s assay were found to underestimate the concentration of protein in the trypsin samples, this time by about 4.5 fold. This problem was also solved by preparing a standard curve using trypsin instead of BSA.
4.3. Determination of inhibition constants for trypsin

4.3.1. Kinetic parameters of trypsin inhibitors

The β-lactams (Table 17) that had been shown to inhibit elastase were also tested for trypsin inhibition. 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) was used as a positive control since this sulphonyl fluoride is a known covalent inhibitor of trypsin. Residual enzyme activity was plotted against time to obtain the observed rate of inactivation ($k_{obs}$) for each concentration of inhibitor from the slope of the line. A typical plot is shown in Figure 68.
Table 17: Structures of compounds tested for inhibition of trypsin

<table>
<thead>
<tr>
<th>Name</th>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td></td>
<td><img src="image" alt="Structure JD260" /></td>
</tr>
<tr>
<td>JD261</td>
<td></td>
<td><img src="image" alt="Structure JD261" /></td>
</tr>
<tr>
<td>JD263</td>
<td></td>
<td><img src="image" alt="Structure JD263" /></td>
</tr>
<tr>
<td>JD264</td>
<td></td>
<td><img src="image" alt="Structure JD264" /></td>
</tr>
<tr>
<td>JD417</td>
<td></td>
<td><img src="image" alt="Structure JD417" /></td>
</tr>
<tr>
<td>JD420</td>
<td></td>
<td><img src="image" alt="Structure JD420" /></td>
</tr>
<tr>
<td>JDx3</td>
<td></td>
<td><img src="image" alt="Structure JDx3" /></td>
</tr>
<tr>
<td>AEBSF</td>
<td></td>
<td><img src="image" alt="Structure AEBSF" /></td>
</tr>
</tbody>
</table>
The rate of association ($k_{ass}$) was then determined from the relationship $k_{ass} = k_{obs}/[I]$ and the values obtained are shown in Figure 69. The $\beta$-lactams were found to inhibit trypsin with rates of association ($k_{ass}$) ranging from about 100 to 700 M$^{-1}$s$^{-1}$. The most potent $\beta$-lactams were once again found to be JD417 (601.37 M$^{-1}$s$^{-1}$), JD260 (709.44 M$^{-1}$s$^{-1}$) and JDx3 (531.37 M$^{-1}$s$^{-1}$). JD420, JD261 and JD264 were not as effective and JD263 (163 ± 275 M$^{-1}$s$^{-1}$) was the least effective. The commercial inhibitor AEBSF that was used as a positive control had $k_{ass}$ values (204 ± 20 M$^{-1}$s$^{-1}$) comparable to JD263 and so was amongst the least effective of the trypsin inhibitors tested.

![Figure 69: $k_{ass}$ of trypsin inhibitors. Errors bars show the standard deviation.](image)

The half-lives ($t_{1/2}$) were then determined from the relationship $t_{1/2} = 0.693/k_{ass}[I]$ and the values are shown in Table 18. At a concentration of 0.3 µM of inhibitor the fastest inhibitors were JD260, JD417 and JDx3. JD417 was the fastest to inactivate trypsin by 50% with a $t_{1/2}$ of 37 minutes, since this compound had a $t_{1/2}$ of 4 minutes against PPE it seems that the $\beta$-lactams are much slower at inhibiting trypsin. JD420 and JD263 reacted more slowly than JD417 since JD420 had a $t_{1/2}$ of 88 minutes.
Among the slowest inhibitors tested were AEBSF and JD264 since JD264 took 633 minutes to inhibit trypsin by 50%.

Table 18: Half-lives (t_1/2) of trypsin inhibitors in minutes

<table>
<thead>
<tr>
<th>Inhibitor (μM)</th>
<th>JD260</th>
<th>JD261</th>
<th>JD263</th>
<th>JD264</th>
<th>JD417</th>
<th>JD420</th>
<th>JDx3</th>
<th>AEBSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>78</td>
<td>60</td>
<td>137</td>
<td>633</td>
<td>37</td>
<td>88</td>
<td>55</td>
<td>203</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>20</td>
<td>N/A</td>
<td>26</td>
<td>28</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>12</td>
<td>50</td>
<td>N/A</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

Values for k_{obs} were then replotted against [I] (Figure 70) in order to obtain values for the rate of inactivation (k_2) and the inhibition constant (K_i) (Table 19).

The β-lactams have k_2/K_i values ranging from 194 M^{-1} s^{-1} to 666 M^{-1} s^{-1} and so are not amongst the most potent trypsin inhibitors although they still quite effectively inhibit trypsin. Once again the data shows that JD260 and JDx3 are the most potent of the β-lactams with JD263 being one of the least effective inhibitors. JD417 had
the strongest affinity for the enzyme with a $K_i$ of 11 mM and JD263 had the least affinity for the enzyme since it had a $K_i$ of 66 mM.

**Table 19: $k_2$ and $K_i$ of trypsin Inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_2$ (sec$^{-1}$)</th>
<th>$K_i$ (mM)</th>
<th>$k_2/K_i$ (M$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td>8</td>
<td>12</td>
<td>666</td>
</tr>
<tr>
<td>JD261</td>
<td>2</td>
<td>11</td>
<td>194</td>
</tr>
<tr>
<td>JD263</td>
<td>3</td>
<td>66</td>
<td>216</td>
</tr>
<tr>
<td>JD264</td>
<td>6</td>
<td>15</td>
<td>411</td>
</tr>
<tr>
<td>JD417</td>
<td>3</td>
<td>11</td>
<td>234</td>
</tr>
<tr>
<td>JD420</td>
<td>7</td>
<td>25</td>
<td>260</td>
</tr>
<tr>
<td>JDx3</td>
<td>7</td>
<td>18</td>
<td>376</td>
</tr>
<tr>
<td>AEBSF</td>
<td>6</td>
<td>28</td>
<td>216</td>
</tr>
</tbody>
</table>

**4.3.1.1. Comparison of inhibitor efficiency as evidenced by UV**

The data consistently reflects that the best trypsin inhibitors are JD260, JDx3 and JD417. These β-lactams had the highest affinity for the enzyme ($K_i$) and JD260 and JDx3 also had the highest rates of inactivation ($k_2$). They also had a high rate of association ($k_{ass}$) and a fast half-life ($t_{1/2}$). Therefore these β-lactams are the fastest and tightest binding trypsin inhibitors amongst the β-lactams tested.

JD420, JD261 JD264 seem to be fairly modest inhibitors compared to the others, the $k_{ass}$, $t_{1/2}$ and the $k_2/K_i$ is neither the highest or the lowest. JD263 was shown to be the least potent trypsin inhibitor as it has the lowest affinity for the enzyme ($K_i$). It was also the slowest at inhibition with a low inactivation rate ($k_2$), low rate of association ($k_{ass}$) and slow half-life ($t_{1/2}$). The commercial trypsin inhibitor AEBSF isn’t much more effective than JD263 since it also has a low rate of association ($k_{ass}$), a slow half-life ($t_{1/2}$) and low value for $k_2/K_i$. Therefore most of the β-lactams were more effective than the commercial inhibitor.
4.4. **ESI-MS of trypsin:Inhibitor complexes**

ESI-MS was also carried out on trypsin after incubation with the β-lactams in an attempt to detect inhibition and identify the mechanism by which inhibition occurs.

4.4.1. **Mechanism of trypsin inhibition by β-lactams**

Since trypsin has the same catalytic machinery as PPE it is possible that each of the β-lactams could inhibit trypsin using the same mechanisms as they did for the inhibition of PPE (mechanism 1 and 2 in Figure 46). LC/MS was used to detect any inhibitor adducts and determined the mechanism of inhibition.

4.4.2. **Detection of covalent inhibition by β-lactams**

Each of the β-lactams (Table 5) was tested for covalent inhibition of trypsin as in 3.4.2. A commercial inhibitor (AEBSF) that was known to covalently inhibit trypsin was used as a positive control.

Trypsin was incubated with AEBSF and a peak was observed (Figure 71) at 23474 that corresponds to trypsin plus 177 Da, which represents the covalent addition of AEBSF to the active site of trypsin. The addition of this peak confirms that AEBSF covalently inhibits trypsin.

Trypsin was then incubated with the β-lactams in the same manner to determine if any covalent inhibition could be detected. If these β-lactams react with trypsin in the same manner as they reacted with PPE then identical inhibitor fragments should be observed (Table 14).

JD260 (Figure 72), JD261 (Figure 73), JD264 (Figure 75) and JD417 (Figure 76) were all found to generate a peak corresponding to the acyl-enzyme intermediate, which is formed via mechanism 2 (Figure 46). The average mass of the fragments can be seen in Table 20. This mechanism is also how these compounds reacted with PPE (3.4.2), therefore we expected trypsin to react in a similar manner.
No covalent adducts were observed after trypsin was incubated with JD263 (Figure 74), which suggests that this inhibitor doesn't inhibit trypsin at all. Since this compound did inhibit PPE this result was unexpected.

JD420 (Figure 77) and JDx3 (Figure 79) were found to generate peaks that closely resemble the expected mass (Table 20) for the formation of the carbinolamine by mechanism 1 (Figure 46). These two compounds also inhibited PPE via this mechanism.

JDx1 (Figure 78) was unable to inhibit trypsin since only the native enzyme could be observed. This compound was not found to inhibit PPE either.
Figure 71: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 10 fold molar excess of AEBSF for 29 hours. The peak at 23297.07 represents the native enzyme and the peak at 23473.71 represents trypsin covalently bound to AEBSF.
Figure 72: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 3 fold molar excess of JD260 for 30 hours. The peak at 23269.69 represents the native enzyme and the peak at 23452.49 represents the stable acyl-enzyme intermediate.
Figure 73: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 10 fold molar excess of JD261 for 24 hours. The peak at 23337.76 represents the native enzyme and the peak at 23587.63 represents the stable acyl-enzyme intermediate.
Figure 74: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 3 fold molar excess of JD263 for 30 hours. The peak at 23315.43 represents the native enzyme.
Figure 75: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 3 fold molar excess of JD264 for 1 minute. The peak at 23281.85 represents the native enzyme and the peak at 23497.36 represents the stable acyl-enzyme intermediate.
Figure 76: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 25 fold molar excess of JD417 for 24 hours. The peak at 23500.86 represents the stable acyl-enzyme intermediate.
Figure 77: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 3 fold molar excess of JD420 for 172 hours. The peak at 23331.91 represents the native enzyme and the peak at 23549.12 represents the carbinolamine.
Figure 78: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 10 fold molar excess of JDx1 for 24 hours. The peak at 23298.75 represents the native enzyme.
Figure 79: Deconvoluted electrospray ionisation mass spectrum after LC/MS of trypsin incubated with a 10 fold molar excess of JDx3 for 24 hours. The peak at 23309.40 represents the native enzyme and the peak at 23524.52 represents the carbinolamine.
Table 20: Size of species resulting from inhibition of trypsin by β-lactams as determined by mass spectrometry. Errors are standard deviations.

<table>
<thead>
<tr>
<th>Name</th>
<th>β-lactam</th>
<th>acyl-enzyme intermediate</th>
<th>Carbinolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Structure</td>
<td>Mass (Da)</td>
<td>Observed Mass (Da)</td>
</tr>
<tr>
<td>JD260</td>
<td><img src="image1" alt="Structure" /></td>
<td>483</td>
<td>181 ± 13</td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image2" alt="Structure" /></td>
<td>533</td>
<td>271 ± 21</td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image3" alt="Structure" /></td>
<td>449</td>
<td>N/A</td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image4" alt="Structure" /></td>
<td>497</td>
<td>230 ± 14</td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image5" alt="Structure" /></td>
<td>410</td>
<td>196 ± 13</td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image6" alt="Structure" /></td>
<td>392</td>
<td>N/A</td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image7" alt="Structure" /></td>
<td>394</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* N/A = Not available
4.4.2.1. Comparison of inhibitor mechanisms

All of the β-lactams inhibited trypsin in the same manner as they did PPE with the exception of JD263, which showed no evidence of inhibition after incubation with trypsin, whereas it did inhibit PPE. The first series of β-lactams inhibitors (JD260, JD261 and JD264) inhibited via mechanism 2 (Figure 46) since they contained the required hydroxyl group present at the C3 position. JD417 also inhibited in this manner since it has the same C3 group as the series 1 β-lactams. JD420 and JDx3, which had no hydroxyl group at the C3 position, inhibited via mechanism 1. JDx1, which showed no evidence of inhibiting PPE, also didn’t inhibit trypsin.

4.4.3. Time dependent inhibition of β-lactams

The β-lactams which were found to covalently inhibit trypsin (4.4.2) were then analysed by LC/MS to determine if they inhibited in a time dependent manner in the same way that they inhibited PPE (3.4.2.1).

JD260 inhibited trypsin in a time dependent manner (Figure 80). Inhibition of trypsin was much slower as compared to inhibition of PPE since it had a $t_{1/2}$ of 1 hour against trypsin and 30 seconds against PPE. However it was the fastest of the β-lactams to inhibit trypsin. Complete inhibition was not observed, the lowest activity detected was 43% after 30 hours incubation with the inhibitor. Reactivation seemed to start after about 50 hours and took more than 3 days to complete.

JD261 was a particularly slow trypsin inhibitor with a $t_{1/2}$ of about 140 hours, by 5 days the enzyme was only about 30% inactivated, and even after 7 days not all of the enzyme has become inactivated yet. Only slight inhibition was observed with JD264 and after 30 hours all of the enzyme had reactivated.

JD417 was the slowest of the β-lactams to inhibit trypsin. After 7 days only 30% of the enzyme had become inactivated and with a $t_{1/2}$ of more than 13 days this was quite a poor inhibitor. JD420 was only marginally better since after 7 days 50% of the enzyme had become inhibited. JDx3 was slightly faster at inhibiting trypsin than
JD417 and JD420 since it had a tₜₜ of about 70 hours and after 7 days the enzyme was 80% inactivated.

Figure 80: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JD260 as evidenced by ESI-MS.

Figure 81: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JD261 as evidenced by ESI-MS.

Figure 82: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JD264 as evidenced by ESI-MS.
Figure 83: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JD417 as evidenced by ESI-MS.

Figure 84: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JD420 as evidenced by ESI-MS.

Figure 85: Graph showing increasing inhibition over time after trypsin was incubated with a 3 fold molar excess of JDx3 as evidenced by ESI-MS.
4.4.3.1. **Comparison of time dependent inhibition by β-lactams**

JD260 was between 70 and 300 times faster at inhibiting trypsin as compared to the other β-lactams since it has a \( t_{1/2} \) of a few hours compared to days. JDx3 and JD261 were slower to inactivate trypsin with a half-lives of about 4 days. JD420 was even slower with a \( t_{1/2} \) of 7 days and JD417 was the slowest with a \( t_{1/2} \) of 14 days. JD264 only slightly inhibited trypsin with the best inactivation observed of 15%.

### Table 21: \( k_{ass} \) and half-lives of β-lactams as evidenced by mass spectrometry

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>( k_{ass} ) (M(^{-1}) S(^{-1}))</th>
<th>( t_{1/2} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td>0.0195</td>
<td>1</td>
</tr>
<tr>
<td>JD261</td>
<td>0.0079</td>
<td>103</td>
</tr>
<tr>
<td>JD264</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>JD417</td>
<td>0.0019</td>
<td>333</td>
</tr>
<tr>
<td>JD420</td>
<td>0.0042</td>
<td>170</td>
</tr>
<tr>
<td>JDx3</td>
<td>0.0085</td>
<td>74</td>
</tr>
</tbody>
</table>

* N/A = Not Available

4.4.4. **Comparison of β-lactam inhibition as evidenced by MS**

The order of potency for the β-lactams from highest to lowest was JD260, JDx3, JD261, JD420, JD417, JD264 and JDx1. JD260 was the most potent β-lactam since it had the fastest \( t_{1/2} \) and the highest \( k_{ass} \). JDx3 and JD261 had slightly slower \( t_{1/2} \) and lower values for \( k_{ass} \) and JD420 and JD417 were less effective at inhibiting trypsin with the slowest \( t_{1/2} \) and smallest values for \( k_{ass} \). JD264 was barely found to inhibit trypsin and JDx1 and JD263 showed no evidence of inhibition.

4.4.5. **Detection of non-covalent inhibition by β-lactams**

Recently there has been a lot of interest in the detection of noncovalent species by MS. Non-covalent complexes have been observed between acyl CoA binding protein...
(ACBP) and various acyl CoA derivatives (Robinson et al., 1996), noncovalent protein/iron interactions have also been observed (Jaquinod et al., 1993) and a review by Joseph A Loo (Loo, 1997) discusses a selection of noncovalent complexes observed by ESI-MS. Electrospray ionisation mass spectrometry has routinely been used to analyse covalent protein/ligand interactions because of its speed and sensitivity for many years now. It has been shown that the gentle ionisation method employed in ESI-MS allows the detection of non-covalent complexes as well.

It was decided to determine if any noncovalent complexes could be observed after trypsin was incubated with the individual β-lactams. In order to observe noncovalent species it is important to maintain the activity of the enzyme during analysis. Therefore the pH and temperature must be carefully controlled. The enzyme:ligand complex was then infused directly into the ESI-MS using low source temperatures and low cone voltages. The samples were also analysed after LC/MS where only covalent intermediates are detected, the spectra from the direct infusion experiment can then be compared to the LC/MS data and any additional peaks are likely to be noncovalent adducts.

A known noncovalent trypsin inhibitor called leupeptin was used as a control (Figure 86). The peak at 23721 represents native trypsin plus 422 Da, which corresponds to trypsin noncovalently bound to leupeptin since the mass of leupeptin is 427 Da. In the same manner noncovalent complexes of JD261 (Figure 87), JD263 (Figure 88), JD264 (Figure 89) and JD417 (Figure 90) were detected.
Table 22: Expected inhibitor species as a result of noncovalent inhibition of trypsin.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>Mass of inhibitor species as determined by MS (Da)</th>
<th>Expected mass of inhibitor species (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leupeptin</td>
<td><img src="image" alt="Structure" /></td>
<td>422</td>
<td>427</td>
</tr>
<tr>
<td>JD260</td>
<td><img src="image" alt="Structure" /></td>
<td>N/A</td>
<td>484</td>
</tr>
<tr>
<td>JD261</td>
<td><img src="image" alt="Structure" /></td>
<td>527</td>
<td>533</td>
</tr>
<tr>
<td>JD263</td>
<td><img src="image" alt="Structure" /></td>
<td>437</td>
<td>449</td>
</tr>
<tr>
<td>JD264</td>
<td><img src="image" alt="Structure" /></td>
<td>476</td>
<td>497</td>
</tr>
<tr>
<td>JD417</td>
<td><img src="image" alt="Structure" /></td>
<td>403</td>
<td>410</td>
</tr>
<tr>
<td>JD420</td>
<td><img src="image" alt="Structure" /></td>
<td>N/A</td>
<td>392</td>
</tr>
<tr>
<td>JDx3</td>
<td><img src="image" alt="Structure" /></td>
<td>N/A</td>
<td>394</td>
</tr>
</tbody>
</table>

*N/A = Not available*
Figure 86: Deconvoluted electrospray ionisation mass spectrum of the direct infusion of trypsin incubated with an equimolar concentration of leupeptin for 15 minutes. The peak at 23298.93 represents the native enzyme and the peak at 23721.29 represents leupeptin non-covalently bound to trypsin.
Figure 87: Deconvoluted electrospray ionisation mass spectrum of the direct infusion of trypsin incubated with a 3 fold molar excess of JD261 for 1 hour. The peak 23293.67 at represents the native enzyme and the peak at 23820.76 represents JD261 non-covalently bound to trypsin.
Figure 88: Deconvoluted electrospray ionisation mass spectrum of the direct infusion of trypsin incubated with a 3 fold molar excess of JD263 for 1 hour. The peak 23294.51 represents the native enzyme and the peak at 23732.29 represents JD263 non-covalently bound to trypsin.
Figure 89: Deconvoluted electrospray ionisation mass spectrum of the direct infusion of trypsin incubated with a 3 fold molar excess of JD264 for 1 hour. The peak 23292.93 at represents the native enzyme and the peak at 23789.01 represents JD264 non-covalently bound to trypsin.
Figure 90: Deconvoluted electrospray ionisation mass spectrum of the direct infusion of trypsin incubated with a 3 fold molar excess of JD417 for 1 hour. The peak 23289.39 represents the native enzyme and the peak at 23692.57 represents JD417 non-covalently bound to trypsin.
This noncovalent inhibition is likely to be the first reversible step (Equation 5) in the inhibition of trypsin before the acylation of the active site serine occurs. The basic mechanism of irreversible inhibition is whereby the enzyme and inhibitor form a reversible EI complex which, after certain time period (dependent on the rate of association), then forms a covalent acyl-enzyme intermediate. Eventually this intermediate will breakdown to release the free enzyme and the modified inhibitor, this occurs a rate dependent on $k_3$. Since the EI complex is reversible and can revert back to the original enzyme and inhibitor it is likely that this is a noncovalent complex. Therefore it is possible that this is the noncovalent species that we have observed.

$$
\begin{align*}
E + I & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} EI & \overset{k_2}{\rightarrow} E-I & \overset{k_3}{\rightarrow} E + I'
\end{align*}
$$

Equation 5: Basic mechanism of irreversible inhibition. The enzyme (E) and Inhibitor (I) initially form a reversible enzyme-inhibitor complex (EI). This complex then forms an acyl-enzyme intermediate (E-I), which breaks down to release the free enzyme (E) and the modified inhibitor (I').

Since the lifetime of the EI complex seems to be significantly longer when analysing trypsin as compared to elastase, perhaps it is only possible to detect this step when the rate of acylation is in the order of hours rather seconds or minutes as in PPE inhibition.
4.4.6. Interpretation of trypsin MS data

The data for the trypsin spectra are not as good as the PPE data and so this makes the interpretation difficult. There are several reasons as to why this data is not as good, it may be that the trypsin sample was not as pure as the PPE samples and so various contaminants could be present in the spectra but this is unlikely since the data in Figure 71 was generated with the same batch of trypsin and this data is extremely good quality. The most likely explanation is that the data was incorrectly deconvoluted by the maxent software, which would have been caused by entering an incorrect value for the peak width at 50% of the peak height. If the peak width that was entered into maxent was smaller than the actual value, this would then result in several thin peaks rather than one broader peak. This explanation also seems likely since in Figure 73 it looks like there are three major peaks at about 23337, 23383 and 23587 Da which are split into three further peaks.
5. **Results and discussion:** Comparison of Elastase & Trypsin

5.1. **Novel $\beta$-lactam preferences**

Studies on the inhibition of PPE by novel $\beta$-lactams (Table 24) have shown that the $\beta$-lactams inhibit elastase and trypsin in a similar manner. The potency rank of the $\beta$-lactams is extremely similar for both enzymes (Table 23), the only real difference is that whereas JD420 is the worst inhibitor of PPE, the worst inhibitor of trypsin is JD263. Although the ranking order of the $\beta$-lactams is very similar for both enzymes, the extent of the inhibition is not.

<table>
<thead>
<tr>
<th>PPE inhibition</th>
<th>Trypsin inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-lactams ranked according to order of potency (most potent first)</td>
<td>$\beta$-lactams ranked according to order of potency (most potent first)</td>
</tr>
<tr>
<td>JD260</td>
<td>JD260</td>
</tr>
<tr>
<td>JDx3</td>
<td>JDx3</td>
</tr>
<tr>
<td>JD417</td>
<td>JD417</td>
</tr>
<tr>
<td>JD261</td>
<td>JD420</td>
</tr>
<tr>
<td>JD264</td>
<td>JD261</td>
</tr>
<tr>
<td>JD263</td>
<td>JD264</td>
</tr>
<tr>
<td>JD420</td>
<td>JD263</td>
</tr>
<tr>
<td>JDx1</td>
<td>JDx1</td>
</tr>
</tbody>
</table>
Table 24: Structures of novel β-lactams

<table>
<thead>
<tr>
<th>Name</th>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD260</td>
<td></td>
<td><img src="image" alt="Structure JD260" /></td>
</tr>
<tr>
<td>JD261</td>
<td></td>
<td><img src="image" alt="Structure JD261" /></td>
</tr>
<tr>
<td>JD263</td>
<td></td>
<td><img src="image" alt="Structure JD263" /></td>
</tr>
<tr>
<td>JD264</td>
<td></td>
<td><img src="image" alt="Structure JD264" /></td>
</tr>
<tr>
<td>JD417</td>
<td></td>
<td><img src="image" alt="Structure JD417" /></td>
</tr>
<tr>
<td>JD420</td>
<td></td>
<td><img src="image" alt="Structure JD420" /></td>
</tr>
<tr>
<td>JDx1</td>
<td></td>
<td><img src="image" alt="Structure JDx1" /></td>
</tr>
<tr>
<td>JDx3</td>
<td></td>
<td><img src="image" alt="Structure JDx3" /></td>
</tr>
</tbody>
</table>
Comparison of the $k_{\text{ass}}$ of the $\beta$-lactams towards both enzymes (Table 25), by both UV and MS, shows that each of the $\beta$-lactams is more specific to PPE than trypsin. The UV results show the average value for $k_{\text{ass}}$ over the range of the 7 inhibitors tested was 13 fold higher for PPE whereas the MS results indicate that the $k_{\text{ass}}$ value is an average of 40,000 fold higher for PPE.

Table 25: Comparison of the $k_{\text{ass}}$ values of PPE and trypsin.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_{\text{ass}}$ (M$^{-1}$ s$^{-1}$) values As determined by UV</th>
<th>$k_{\text{ass}}$ (M$^{-1}$ s$^{-1}$) values As determined by MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPE</td>
<td>Trypsin</td>
</tr>
<tr>
<td>JD260</td>
<td>9875</td>
<td>709</td>
</tr>
<tr>
<td>JD261</td>
<td>6032</td>
<td>318</td>
</tr>
<tr>
<td>JD263</td>
<td>2597</td>
<td>163</td>
</tr>
<tr>
<td>JD264</td>
<td>4334</td>
<td>255</td>
</tr>
<tr>
<td>JD417</td>
<td>5001</td>
<td>601</td>
</tr>
<tr>
<td>JD420</td>
<td>1143</td>
<td>351</td>
</tr>
<tr>
<td>JDx3</td>
<td>8790</td>
<td>531</td>
</tr>
<tr>
<td>Average increase in PPE $k_{\text{ass}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comparison of the $t_{1/2}$ of the $\beta$-lactams towards both enzymes (Table 26) was carried out for both the UV and the MS data. Both sets of data show that the $\beta$-lactams are considerably faster at inhibiting PPE than trypsin. The UV data shows the $\beta$-lactams are an average of 16 times faster at inhibition of PPE. Whereas the MS data shows that the $\beta$-lactam are an average of 5000 times faster at inhibiting PPE.
Table 26: Comparison of the $t_{1/2}$ of PPE and trypsin.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$t_{1/2}$ (hrs) values \textit{As determined by UV}</th>
<th>$t_{1/2}$ (hrs) values \textit{As determined by MS}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin</td>
<td>PPE</td>
</tr>
<tr>
<td>JD260</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>JD261</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>JD263</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>JD264</td>
<td>11</td>
<td>0.2</td>
</tr>
<tr>
<td>JD417</td>
<td>0.6</td>
<td>0.07</td>
</tr>
<tr>
<td>JD420</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>JDx3</td>
<td>1</td>
<td>0.07</td>
</tr>
<tr>
<td>Average increase in $t_{1/2}$ for trypsin</td>
<td>16 ± 18</td>
<td></td>
</tr>
</tbody>
</table>

Although the MS and UV results are not consistent with one another in terms of the values for $k_{\text{ass}}$ and $t_{1/2}$, they both indicate that the $\beta$-lactams are more specific for PPE than trypsin although to what extent is unclear. The UV results suggest that the $\beta$-lactams are about 15 fold faster at inhibition of PPE as compared to trypsin, whereas the MS results suggest that the value could be between 5,000 and 40,000.
5.2. Comparison of UV & MS in measuring inhibition

Mass spectrometry is primarily used in a qualitative manner to identify the presence or absence of a compound. We were interested to determine if it could also be used in a quantitative manner to determine for example, the extent of enzyme inhibition and perhaps even carry out some enzyme kinetics. Similar experiments using ESI-MS and UV-visible spectrometry were carried out and the results were compared in order to determine if mass spectrometry could be used to quantify enzyme inhibition.

5.2.1. Elastase

The UV data for PPE inhibition consistently shows that the β-lactams in order of most potent to least are JD260, JDx3, JD417, JD261, JD264, JD263 and JD420. The MS data indicates that the ranking is JD260, JDx3, JD264, JD261, JD417, JD263 and JD420. With JDx1 showing no evidence of inhibition. Therefore the UV and MS data agree on the two best inhibitors and the two worst inhibitors, but the there seems to be some discrepancies concerning the order of the three β-lactams in-between. UV and MS were both used to calculate the $k_{ass}$ of the inhibitors, the UV values were considerably higher than the MS results for each of the β-lactams.

5.2.2. Trypsin

The UV results show that the β-lactams in order of trypsin inhibition with the most potent first is JD260, JDx3, JD417, JD420, JD261 JD264 and JD263. The MS results consistently show that the ranking order is JD260, JDx3, JD261, JD420, JD417, JD264 and JD263. Again the UV and MS results agree on the two best and two worst inhibitors but the order of the three in-between is unclear. The values for $k_{ass}$ are also considerable higher (> 3 x 10^4 fold) when determined by UV.

5.2.3. Conclusion

The discrepancies between the two sets of results may be due to the different sets of conditions used. In order to detect enzyme inhibition by MS, protein concentrations of 100 μM were employed. It was impossible to use the same protein concentration
for UV since large quantities of substrate would be required. Therefore a protein concentration of 100 nM was used. Since the same concentration of enzyme and inhibitor could not be used in both experiments, the enzyme/inhibitor ratio was kept constant with typically a 3 fold excess of inhibitor being used. These concentration differences may have had an effect on the validity of comparing the data sets.

Another factor that may be important is that in the UV studies enzyme inhibition is monitored in the presence of a chromogenic substrate whereas in the MS studies this is not required. In order to check whether the absence of the chromogenic substrate effects the validly of the MS data, the chromogenic substrate can be added to the sample and analysed by ESI-MS. Aplin et al., (Aplin et al., 1994) carried out a similar experiment whereby they added a chromogenic substrate to the enzyme and identified a peak corresponding to the hydrolysed substrate. They also repeated the experiment in the presence of an irreversible PPE inhibitor and no hydrolysis was observed since the inhibitor was preventing PPE from reacting with the substrate.

5.3. Errors

Although the data in this thesis give a good indication as to the general trends of the β-lactams in the inhibition of PPE and trypsin, it is clear that the errors are quite large and so great care must be taken not to over interpret the data. The most likely cause for these errors is the weighing out of enzymes since stock solutions of PPE and trypsin were 2.6 mg/ml and 2.3 mg/ml respectively. These were weighed out on 4 figure balance but it would be preferable to use either a 6 figure balance or scale the study up so that the errors in weighing would be minimal.
6. **Results and discussion: Future Work**

The β-lactams JD260 and JD417 (Figure 91) are structurally very similar differing only in the leaving group at the R² position (Figure 92). Despite this similarity JD260 is about twice as effective as JD417 against PPE and the covalently bound E-I species seems to be more stable. Therefore it would be interesting to obtain the X-ray crystal structure of the acyl fragment formed after the inhibition of PPE by JD417 to determine if there are any major differences in the way it behaves as compared to the X-ray crystal structure of JD260. Co-crystallisation studies on the first series of β-lactams (JD260-JD264) were previously carried out by Violet Anderson, during her PhD at the University of Edinburgh (Anderson, 2001).

![Figure 91: The structures of JD260 and JD417](image)

It would also be interesting to determine if the noncovalent complexes, observed after the β-lactams were incubated with trypsin, could be identified by X-ray crystallography to determine if they are enzyme-inhibitor complexes as suggested.

The β-lactams have shown how compounds with different groups at the C3 and the R¹ position (Figure 92) affect the inhibition of PPE and trypsin. It would be interesting to develop a series of β-lactams with different groups at the R² position to determine if the leaving group has any considerable effect on inhibition. It was originally thought that the structure of the leaving group was of minimal importance.
since it is quickly eliminated upon acylation of the active site serine. Studies on JD260 and JD417 (Figure 91), which are identical except for the structure of their leaving group, gave unexpected results. It was thought that these compounds would behave very similarly but we have shown that JD260 is more potent and so perhaps the leaving group is more important than originally anticipated.

![Figure 92: Basic structure of a monocyclic β-lactam.](image)

Regarding the specificity of the β-lactams it would be interesting to determine if they have any activity towards proteases outwith the serine protease family, such as members of the cysteine, aspartic or metallo proteases. Since specific inhibitors are normally much more useful therapeutically.

Concerning the β-lactams as therapeutic drugs, the oral activity of the β-lactams could be tested to determine if any are stable in the blood and plasma and so would be viable inhibitors for clinical trials. This could be established by administering the drug orally to an animal subject, the concentration of elastase in the blood could then be measured over time (Macdonald et al., 2001). This would determine if the compound caused any decrease of elastase in the blood or plasma. It would also determine the length of time it took to return to the original level of elastase and so indicate how potent the drug is \textit{in vivo}.
7. Bibliographic references


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