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Mechanism-based inhibition of HsaD: a C-C bond hydrolase essential for survival of *M. tuberculosis* in macrophage

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Abstract

M. tuberculosis remains the leading cause of bacterial mortality worldwide. With the increasing prevalence of multidrug resistant organisms identification of novel targets for anti-tuberculars remains a priority. 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (HsaD), part of the cholesterol metabolism operon, is vital for survival within macrophage. The C-C-bond hydrolase, HsaD has a serine protease-like catalytic triad in the active site. We therefore wished to test a range of known serine protease and esterase inhibitors for their effects on HsaD activity. As well as providing a potential starting point for drug development, the mechanism based inhibition approach provides evidence on the mechanism of C-C bond hydrolysis. This screen also provides a route to initiate development of fragment based inhibitors.

Key words: HsaD, *M. tuberculosis*, inhibitors, mechanism, MCP-hydrolase, cholesterol.

Introduction

Although *M. tuberculosis* has been almost eradicated in the developed world around 1.4 million people died from the disease in 2011 [1] (95% were in developing countries) and 8.7 million people became infected. Around 3.4% of all cases were multi-drug resistant (MDR-TB) tuberculosis (defined as those with resistance to rifampicin and isoniazid) while there were around 25,000 cases of extremely drug resistant (defined as those MDR-TB which are also resistant to fluroquinolone and a second line antitubercular e.g. amikacin).

The vital role of cholesterol in the infection cycle of *M. tuberculosis* is becoming increasingly apparent [2]. Cholesterol is vital for phagocytosis of *M*. tuberculosis by macrophage [3] and also plays an important role as an energy source during bacterial survival within macrophage [4]. The cholesterol metabolism operon of *M. tuberculosis* has been identified and includes the genes *HsaA-D* [4]. Gene deletion mutants of *HsaC* and *HsaD* have shown that these enzymes are required for survival inside macrophage [5]. As *HsaD* is an essential gene for survival inside macrophage it is a promising target for anti-tubercular therapy.

HsaD is a member of the meta-cleavage product (MCP)-hydrolase class of enzymes which are a subfamily of the α/β hydrolases [6]. HsaD catalyses the cleavage of 4,9-DHSA within the cholesterol metabolism pathway [4]. HsaD cleaves carbon-carbon bonds via a serine protease-like catalytic triad [6,7].

Three classes of inhibitors were tested for activity against HsaD (Sup. Fig. 1). The largest group were serine protease inhibitors. A number of covalent inhibitors e.g. phenylmethylsulfonyl fluoride (PMSF), were tested alongside non-covalent inhibitors e.g. benzamidine. Acetylcholinesterases are also members of the α/β hydrolase family and catalyse their reactions via a serine protease-like catalytic triad[8]. A range of acetylcholinesterase specific inhibitors were also tested e.g. neostigmine. Humans have a structural homologue of HsaD called monoglyceride lipase (MGL [9]). Like acetylcholine esterases

it shares the same overall fold as HsaD and also acts via a serine protease like catalytic triad. A number of MGL specific inhibitors have been tested e.g. cholesterol-like pristimerin [10]. Due to the hydrophobic nature of the physiological substrates of MGL and HsaD it seemed likely that similar inhibitors may be favoured by both enzymes. We describe the effect of these inhibitors and the results shed light on the mechanism of the enzyme and provide a basis for future approaches to inhibitor design.

Materials and Methods

All reagents were obtained from Sigma Aldrich unless specified. The structures of all inhibitors are provided in supplementary figure 1. DCI (3,4-dichloroisocoumarin) was obtained from Calbiochem, JLK6 from Tocris Biosciences and NAM (N-arachidonyl maleimide) was from Cayman Chemicals. All inhibitors were dissolved in DMSO except NAM which was obtained dissolved in ethanol. HOPDA was synthesised as described previously [11] by Almac Sciences and dissolved in ethanol as it proved to be more stable in ethanol than DMSO (Sup. Fig. 2).

HsaD was expressed in *P. putida* KT2442 and purified as described previously [11]. Enzymatic activity was measured via monitoring OD_{450} on a Sunrise plate reader (Tecan). ε_{450} of HOPDA was measured as 13,200 $M^{-1}cm^{-1}$. All reactions were carried out in 100 mM phosphate buffer pH 7.5, 20 mM NaCl, 5% (v/v) DMSO, 1 % (v/v) ethanol. All inhibitors were incubated at 21°C with HsaD for 20 minutes unless otherwise stated.

Mass spectroscopy was carried out via electrospray ionisation time of flight mass spectroscopy using a LCT mass spectrometer (Micromass).

Results
Serine protease inhibitors

PMSF is a very broad spectrum serine protease inhibitor that forms a covalent adduct with the catalytic serine and has previously been shown to inhibit members of the MCP-hydrolase family [12,13]. PMSF showed relatively weak inhibition of HsaD, with an IC_{50} of 630 μM after 20 minutes incubation (Figure 1A). As well as PMSF a range of other serine protease inhibitors have also been tested including, 4-amidinophenylmethanesulfonyl fluoride (APMSF), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), benzamidine, DCI and JLK6 (Fig. 1B). APMSF, a close relative of PMSF, showed significantly poorer inhibition than PMSF (Fig. 1A & B) indicating that addition of the positively charged amidino group has a detrimental effect on binding. A third sulfonyl fluoride based inhibitor, AEBSF also inhibited HsaD poorly (Fig. 1B).

Like PMSF, DCI is known to be a broad spectrum covalent inhibitor of serine proteases [14], although their chemical structures are unrelated. DCI showed the strongest inhibition of all compounds tested with an IC_{50} of 17 μM (Fig. 1C). Covalent modification of HsaD by DCI was shown via mass spectrometry to increase the molecular weight of HsaD by an amount consistent with a single covalent modification by DCI i.e. (Sup. Fig. 3). The structural homologues of DCI, JLK-6 and S920428 showed significantly poorer inhibition (Figure 1B & C). The non-covalent inhibitors including benzamidine, leupeptin and nafamostat mesylate also showed weak inhibition of HsaD (Fig. 1B) compared to PMSF and DCI.

MGL and acetylcholine esterase inhibitors

MGL like HsaD catalyses the turnover of highly hydrophobic substrates, as such the inhibitors that have been identified tend to be insoluble (e.g. pristimerin and NAM). Although pristimerin is the most active non-covalent inhibitor tested (35% inhibition at 50 μM – Fig. 2A) further investigation was hampered by its poor aqueous solubility under conditions which are required for HsaD to remain active.

NAM and JZL184 are covalent inhibitors: JZL184 like DCI and PMSF modifies the catalytic serine of MGL [15], whilst NAM modifies a cysteine in the active site of MGL [16]. Compatible with the lack of a cysteine residue in the active site of HsaD (Fig. 2A). JZL 184 proved a better inhibitor (Fig. 2A) but was difficult to work with due to its hydrophobic nature and hence poor solubility.

A series of specific acetylcholine easterase inhibitors were tested for inhibition of HsaD (Fig. 2B). These included eserine, edrophonium, tacrine, neostigmine, pyridostigmine and trichlorfon. After incubation with HsaD, trichlorfon inhibited poorly. Eserine and neostigmine show better inhibition but still not as strong as was observed with DCI (~30% inhibition at 1 mM). The other acetylcholinesterase inhibitors did not significantly inhibit HsaD.

Discussion

Two mechanisms have been proposed for the hydrolysis of substrates by MCP-hydrolases. The first is based on the mechanism known to occur in serine proteases and proceeds via an acyl-enzyme and tetrahedral intermediate [17]. The second requires a keto-enol tautomoerisation resulting in a gem-diol intermediate [18]. Recent mutagenesis experiments combined with structural studies resulted in trapping of the acyl-enzyme intermediate of HOPDA hydrolysis, by another member of the C-C bond hydrolase family, BphD [17] strongly supporting the first mechanism. Inhibition by PMSF and DCI are also consistent with this mechanism as both PMSF and DCI act as tetrahedral and acyl-enzyme intermediate analogues respectively when they modify the active site serine.

The most successful inhibitors were those that covalently modify HsaD (e.g. DCI). The primary issue with DCI and other covalent inhibitors tends to be their broad specificity profile making them poor starting points for inhibitor design. To help understand the specificity observed among the covalent inhibitors, the structure of HsaD modified with PMSF was solved (data not shown). Although density was observed for the sulphonate group covalently linked to Ser114, there was insufficient density to accommodate the phenylmethyl group of PMSF. The structure of another α/β hydrolase fold protein (RsbQ) has been solved modified with PMSF [19]. A comparison of the active sites of RsbQ and HsaD is shown in figure 3. In contrast to the small hydrophobic active site of RsbQ (Fig. 3A), HsaD has a large open active site (Fig. 3B). The RsbQ active site is perfect for binding the hydrophobic phenylmethyl group of PMSF as it is bordered by three phenylalanine residues. The more open site of HsaD means that PMSF is more mobile explaining the lack of density for the phenylmethyl group. The hydrophobic nature of the active site close to the catalytic serine (Fig. 3B) makes binding of the positively charged amidino group of APMSF unfavourable and explains its relatively poor inhibition compared to PMSF (Fig. 1A).

It is interesting to consider that those inhibitors with the broadest specificity against serine proteases and acetylcholine esterases are also the inhibitors which show the best inhibition against HsaD. PMSF and DCI inhibit a wide range of serine proteases e.g. thrombin, elastase and trypsin [14,20], both also inhibit acetylcholine esterase [14,20] and PMSF inhibits MGL [21], thus it is unsurprising that they also inhibit HsaD. More selective serine protease inhibitors such as APMSF (does not inhibit either chymotrypsin or acetylcholine esterase [22]) do not inhibit HsaD. The acetylcholine esterase inhibitors *e.g.* eserine, are drug molecules and thus designed to show very good specificity for acetylcholine esterase, thus it is unsurprising that they poorly inhibit HsaD.

The majority of the non-covalent inhibitors showed very poor inhibition of HsaD. This is not surprising as the main anchor for covalent inhibitors is the active site serine whereas the non-covalent inhibitors are dependent upon the shape/charge distribution of the active site. Poor inhibition by the majority of non-covalent inhibitors (e.g. benzamidine) can be linked to their relatively small size. HsaD has a large open active site (Fig. 3B) which is considerably larger than that of either serine proteases or acetylcholinesterases (e.g. trypsin – Fig. 3C). The active site of MGL is more comparable in size to that of HsaD (Fig. 3D). Non-covalent inhibitors of MGL are thus significantly larger than those of serine protease (e.g. compare pristimerin and benzamidine – Sup. Fig. 1) and fill more of the HsaD active site and thus have lower IC_{50} values.   The lipophilicity of the inhibitors also has a direct effect with the more hydrophobic inhibitors e.g. pristimerin being favoured over charged ones e.g. neostigmine, due to the apolar nature of the HsaD active site. Further work on the hydrophobic non-covalent inhibitors is hampered though by their poor solubility profiles.

The aim of this project is to form a starting point for fragment based drug design [23]. DCI makes a useful starting point for the design of a covalent inhibitor due its low IC_{50} value (Fig. 1A), it is however limited in its usefulness due to its ability to inhibit a broad range of enzymes [14]. Structural studies are on-going to determine the mode of binding of DCI within the active site in order to conduct structure based inhibitor design to take advantage of the unique chemical environment of the active site (Fig. 3B) in order to generate specificity. Among the non-covalent inhibitors pristimerin was found to be the most potent (Fig. 2A). The most potent non-covalent inhibitors of HsaD identified during this study are difficult to work with due to their poor aqueous solubility, as a result further screening for more polar non-covalent small molecule inhibitors will be required for lead discovery.

Figure Legends

Fig 1: Inhibition of HsaD by serine protease inhibitors. (A) Comparing inhibition of HsaD by PMSF and APMSF. (B) Comparison of serine protease inhibitors. All compounds in blue were tested at 1 mM while all compounds in purple were tested at 100 μM. All covalent inhibitors were incubated for 20 minutes with HsaD prior to addition of HOPDA. (C) Comparing inhibition of HsaD by DCI and JLK-6. All tests were carried out in triplicate and error bars show the standard deviation of the readings. Curve fitting was carried out in Graphpad Prism.

Fig 2: Effect of inhibitors of other MCP-hydrolases. A) Inhibition of HsaD by MGL inhibitors. B) Inhibition of HsaD by acetylcholinesterase inhibitors. All compounds in red were tested at 1 mM, those in blue at

100 μM and those in green at 50 μM. All experiments were carried out in triplicate and error bars show the standard deviation of the readings.

Figure 3: comparing the sizes of the binding sites of various enzymes. (A) binding of PMSF to the catalytic site of RsbQ. (B & C) binding of PMSF to HsaD and MGL respectively modelled on its binding to RsbQ. (C) binding of PMSF to trypsin. The structure of HsaD was taken from PDB 2VFC [6], while RsbQ is from PDB 1WPR [19], trypsin from PDB 1PQA [24] and MGL from PDB 3JW8 [7]. RMSD of overlay between RsbQ and HsaD was 2 and to MGL was 2.1 via secondary structure matching. In B-D PMSF is shown with green carbon atoms while in (A) the three interacting phenylalanine residues are labelled. The surfaces shown in (B)-(D) are coloured by contact potential. Structures were visualised and aligned in CCP4MG [25].

Sup. Fig. 1: Structures of the inhibitors used during this study. (A) PMSF, (B) APMSF, (C) benzamidine, (D) AEBSF, (E) DCI, (F) JLK6, (G) NFM, (H) leupeptin, (I) S920428, (J) tacrine, (K) neostigmine, (L) edrophonium chloride, (M) pyridostigmine, (N) eserine,(O) JZL 184, (P) trichlorfon, (Q) pristimerin, (R) NAM.

Sup. Fig. 2: stability HOPDA dissolved in ethanol (A) and DMSO (B). HOPDA was prepared as a 20 mM stock in either ethanol or DMSO. Aliquots were stored at either 4°C or 25°C and diluted in phosphate buffer to a final concentration of 100 μM.

Sup. Table 1: Molecular weight of HsaD determined by mass spectroscopy before and after modification with DCI. Molecular weight was predicted based upon amino acid sequence using protparam from expasy (http://web.expasy.org/protparam/).

Abbreviations:

4,9-DHSA: 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid. AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride. APMSF: 4-amidinophenylmethanesulfonyl fluoride. DCI: 3,4-dichloroisocoumarin. DMSO: dimethylsulfoxide. HsaD: 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase. MCP: meta-cleavage product. MGL: monoglyceride lipase. NAM: N-arachidonyl malemide. PMSF: phenylmethylsulpfonyl fluoride.

Acknowledgements

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