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## Susceptibility and mode of binding of the *Mycobacterium tuberculosis* cysteinyl transferase mycothiol ligase to tRNA synthetase inhibitors

Maria-Teresa Gutierrez-Lugo<sup>†</sup>, Carole A. Bewley<sup>\*</sup>

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, United States

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### ABSTRACT

The cysteinyl transferase mycothiol ligase, or MshC, catalyzes the fourth step in the biosynthesis of the small molecular weight thiol mycothiol. MshC is essential for growth of *Mycobacterium tuberculosis*. Two groups of known aminoacyl tRNA synthetase inhibitors were evaluated for inhibition of *M. tuberculosis* MshC including aminoacyl adenosine analogs and natural products. Using enzyme assays, isothermal titration calorimetry and NMR, we show that MshC is selectively inhibited by cysteinyl sulfamoyl adenosine, and that discrimination occurs at the amino acid moiety.

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Mycothiol ligase, also referred to as MshC, is an enzyme essential for growth of *Mycobacterium tuberculosis*<sup>1,2</sup> (Mtb) making it an attractive target for tuberculosis drug development.<sup>1,3,4</sup> MshC catalyzes the ATP-dependent condensation of cysteine and glucosamine- $\alpha$ -(1-1)-myo-D-inositol (GlcN-Ins) to form cysteinyl GlcN-Ins (Cys-GlcN-Ins) (Fig. 1), an intermediate in the biosynthesis of mycothiol (AcCys-GlcN-Ins). In mycobacteria, mycothiol functions as a redox buffer and as a reserve of cysteine. Mycothiol is also involved in the detoxification of antibiotics and reactive oxygen and nitrogen species.<sup>3</sup>

MshC was first purified from *Mycobacterium smegmatis* and shown to be the product of *cysS2*,<sup>5</sup> a gene originally annotated as a second cysteinyl-tRNA synthetase (CysRS) in the Mtb genome.<sup>6</sup> MshC from Mtb shares ~35% sequence homology with CysRS from Mtb and *Escherichia coli* (Fig. 2). Based on sequence homology and similarity of the reactions these enzymes catalyze, a two-step catalytic mechanism was proposed for MshC.<sup>5</sup> The first step, common to CysRS and MshC, includes adenylation of cysteine by ATP to form the activated cysteinyl-AMP (Cys-AMP) intermediate. For CysRS, the second step involves transfer of cysteine to the ribose unit at the 3' terminus of tRNA<sup>Cys</sup> to produce the charged cysteinyl ester.<sup>7</sup> By comparison the second step for MshC involves amide bond formation between the activated carbonyl carbon of Cys-AMP and the free amino group of GlcN-Ins to form

Cys-GlcN-Ins (Fig. 2).<sup>5</sup> In addition, kinetic and mechanistic characterization of *M. smegmatis* MshC revealed that MshC follows a 'bi uni bi ping-pong' steady-state kinetic mechanism where ATP and then Cys are bound by MshC to generate the cysteinyl-AMP intermediate and pyrophosphates, and release of pyrophosphates occurs prior to binding and transfer of GlcN-Ins to Cys-AMP.<sup>8</sup>

The crystal structure of *M. smegmatis* MshC in complex with the bi-substrate analog 5'-O-[N-(L-cysteinyl) sulfamoyl] adenosine (**1**, CysSA) was recently reported.<sup>9</sup> In addition to revealing MshC to be structurally homologous to CysRS with the catalytic domain possessing a Rossmann fold, the structure showed that the thiol group of **1** interacts with a zinc ion at the base of the active site suggesting that it participates in amino acid binding and discrimination as shown previously for *E. coli* CysRS.<sup>10</sup> Despite its utility as an antitubercular target, little is known about small molecule inhibitors of MshC. *E. coli* CysRS is known to be highly selective for activation of Cys, discriminating against its serine isostere by more than 20,000-fold at the binding step due in part to the higher affinity of the zinc-thiolate interaction over the zinc-hydroxyl interaction.<sup>10,11</sup>

To investigate susceptibility of MshC to known aminoacyl tRNA (aa-tRNA) synthetase inhibitors, we evaluated the effects of two types of inhibitors toward MshC. These included the three aminoacyl adenosine analogs CysSA (**1**), SerSA (**2**) and AspSA (**3**), and four natural product inhibitors ochratoxin A (**4**), borrelidin (**5**), cispentacin (**6**), and mupirocin (**7**) (Fig. 3). Compounds **1–3** are mechanistic inhibitors of the first catalytic step of CysRS, SerRS, and AspRS, respectively,<sup>12</sup> and the natural product inhibitors mimic to varying extents substrates and/or intermediates of

<sup>\*</sup> Corresponding author. Tel.: +1 301 594 5187; fax: +1 301 402 0481.

E-mail address: [caroleb@mail.nih.gov](mailto:caroleb@mail.nih.gov) (C.A. Bewley).

<sup>†</sup> Present address: Division of Therapeutic Proteins, Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, MD, United States.

Mtb. MshC	MQSWYCPVPVFLPGRGPGQLRLYDSADRQVRVPVAPGSK---ATMYVCGITPYDATHLGHAA	58
Msmeg. MshC	MQWSWAPAIPIVVPVGRGPALRLFDSDADRQVRPVPFGPT---ATMYVCGITPYDATHLGHAA	58
Mtb. CysS	-----MTDRARLRRLHDTAAGVVRDFVFLRPGHVSIIYLGATVQGLPHIGHVRS	48
E.coli CysS	-----MLKIFNTLTRQKEEFKPIHAGEVMGYCGITVYDCLHIGHGRT	43
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Mtb. MshC	YVTFDLIHLRLWLDLGHLEHYQNIITDIDDLPLFERADRQDGVWRDLAQAEVALFCEDMAAL	118
Msmeg. MshC	YLTFDLVHLRLWLDAGHTVQYVQNVITDVDDPLFERAERDGDIDWRTLGDRETQLFREDMAAL	118
Mtb. CysRS	GVAFDILRRWLLARGYDVAIFIRNVITDIEDKILAKAAAAGRPPWWEWAATHERAFTAAYDAL	108
E.coli CysRS	FVAFDVVARYLRFLGYKLYVRNITDIDDKIKKANENGESFVAMVDRMIAEMHKDFDAL	103
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Mtb. MshC	RVLPPQDYVGATEAIAEMVELIEKMLACGAAYVIDREMGEYQDIYFRADATLQFGYESGY	178
Msmeg. MshC	RVLPPHYVAATDAIAEVVMEVKLLASGAAYIV---EDAEPDVYFRADATAQFGYESGY	176
Mtb. CysRS	DVLPPSAEPRATGHITQMIEMIERLIQAGHAYTG-----GGDVYFDVLSYPEYQGLSGH	162
E.coli CysRS	NILRPDMEPRATHHIAEIIELTEQLIAKHAYVAD-----NGDVMFDPVTPPTYGVLSRQ	158
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Mtb. MshC	DRDTMLRLCEERGGDPRRPGKSDLDALLWRAARPGEPSWPSPFGPGRPGWHVECAIAL	238
Msmeg. MshC	DRDTMLTLFAERGGDPDRPGKSDLDALLWRAARPGEPSWPSPFGPGRPGWHVECSAIAL	236
Mtb. CysRS	K-----IDDVHGE---GVAAGKRDQDFTLWKGKPGEPSWPTFWRGRPGWHLECSAMAR	216
E.coli CysRS	D----LDQLQAGARVDVDDKRNPMDFVLWKMSKEGEPSPWSPFWGAGRPGWHIECSAMNC	214
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Mtb. MshC	SRIGSGLDIQGGGSDLIFPHEFHFAAAECVSGERRFARHYVHAGMIGWDGHKMSKSRGN	298
Msmeg. MshC	TRIGTGLDIQGGGSDLIFPHEHYAAHAESVTGERRFARHYVHTGMIGWDGHKMSKSRGN	296
Mtb. CysRS	SYLGPEFDIHCGGMDLVFPHEHIIAQSRAGADG---FARYLWNGMIVTMGEEKMSKSLGN	274
E.coli CysRS	KQLGNHFDIHGGGSDLMFPHENIIAQSTCAHDGQ-YVNYWMHSGVMVDREKMSKSLGN	273
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Mtb. MshC	LVLVSALRAQDVEPSAVRLGLLAGHYRADRFWSQQVLDEATARLHRWRTATALP----AG	354
Msmeg. MshC	LVLVSQRLAQGDVPSAIRLGLFSGHYREDRFWSNEVLDEANARLARWRSATALP----EA	352
Mtb. CysRS	VLSMPAMLQR-VRPAELRYLYLSAHYRSMLEFSETAMQDAVKAYVGLDFLHRVTRVGA	333
E.coli CysRS	FFTVDVLKY-YDAETVRYFLMSGHYRSLQNLSEENLKQARAALERLYTALRGTDKTVAP	332
	. . : * *::: * * *::: *::: *::: *::: *	
Mtb. MshC	PAADVVARVRRYLADDLDTFKAIAALDGWVTDAVE-YGGHDAGAPKLVATAIDALLGVD	413
Msmeg. MshC	PDATDVITARVQYLADDLDTFKAIAALDGWCTDALS-YGGHDTESPRLVATTVDALLGVD	411
Mtb. CysRS	VCPGDPTPRFAALDDDLSPVIALAEIHVRAEGNRALDAGDHGALRSASATRAMMGIL	393
E.coli CysRS	AGGEAFEAFRIEAMDDDFNTPEAYSVLFDMAREVNR-LKAEDMAAANAMASHLRKLSAVL	391
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The reaction scheme illustrates the synthesis of Cys-Glc-ins. It begins with Cys (cysteine) reacting with ATP to form Cys-AMP and release of PPi. Cys-AMP then reacts with Glc-ins (glutathione-insulin complex) to form Cys-Glc-ins and release of AMP.

other aminoacyl adenosines. Though unrelated structurally, mupirocin and isoleucyl sulfamoyl adenosine (IleSA) bind IleRS similarly with the epoxy, tetrahydropyran and C-13 hydroxyl of mupirocin occupying the same regions as the phosphate, ribose and isoleucine moieties of Ile-AMP, respectively.<sup>12,13</sup> Borrelidin inhibits *E. coli* ThrRS at the step of threonine activation in a non-competitive manner with respect to threonine and ATP, yet was suggested to bind at the back of the active site to disrupt zinc ion coordination and ThrRS activity.<sup>14</sup> Ochratoxin A contains a phenylalanine unit and inhibits *Thermus thermophilus* PheRS.<sup>12</sup> Interestingly, when the phenylalanine of ochratoxin is replaced by valine, the resulting compound inhibits ValRS.<sup>12</sup> The proline analog cispentacin [(1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid] weakly inhibits *Candida albicans* ProRS, and a closely related analog icofungipen [(1*R*,2*S*)-2-amino-4-methylidene-cyclopentane] inhibits *C. albicans* IleRS.<sup>15</sup>

previously.<sup>16</sup> The results are shown in Table 1. Of the known aa-tRNA synthetase inhibitors, only CysSA had an inhibitory effect on the activity of MshC at reasonable concentrations, showing an IC<sub>50</sub> value of 50 nM. These data suggest that MshC shows comparable discrimination for thiol-containing inhibitors, similar to CysRS. The remaining six compounds showed no effect until doses reached mM concentrations.

Data from inhibition assays employing compounds **2** and **3** nevertheless gave standard dose–response curves showing no signs of aggregation or precipitation at mM concentrations. Thus we next used isothermal titration calorimetry (ITC) to measure equilibrium association constants, and Saturation Transfer Difference (STD)<sup>17</sup> NMR and NOESY or transferred NOESY (trNOESY) experiments to compare the mode of binding of CysSA versus AspSA. First, ITC measurements showed CysSA to bind MshC with a  $K_D$  of 48.5 nM, comparable to the observed 50 nM  $IC_{50}$  value. trNOESY spectra of CysSA free in solution and in the presence of MBP–MshC further confirmed binding as cross peaks changed in sign from positive to negative, respectively ([Supplementary](#), Figs. S1–2). In contrast NOESY spectra of AspSA in the presence of MBP–MshC did not

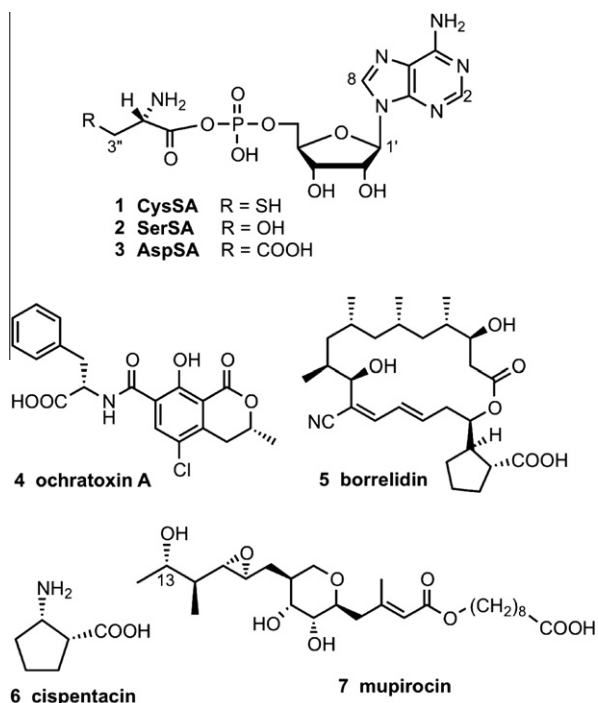


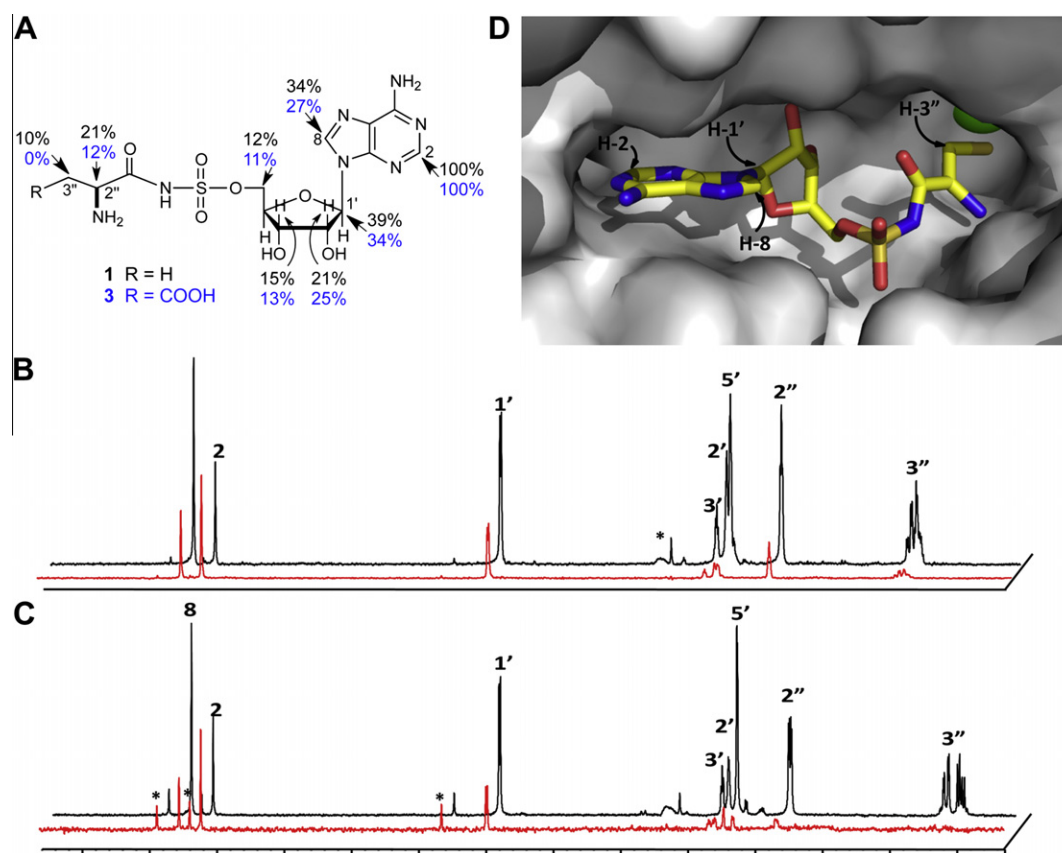
Figure 3. Known tRNA synthetase inhibitors.

**Table 1**  
 Inhibition of MshC

Compound	IC <sub>50</sub>	Aminoacyl-t-RNA synthetase
<b>1</b> CysSA	50 nM	CysRS
<b>2</b> SerSA	5 mM	AspRS
<b>3</b> AspSA	1.2 mM	SerRS
<b>4</b> Ochratoxin A	2.5 mM	PheRS
<b>5</b> Borrelidin	>2 mM	ThrRS
<b>6</b> Cispentacin	>4 mM	ProRS
<b>7</b> Mupirocin	>5 mM	IleRS

contain any cross peaks (SI, Fig. S3) suggesting absence of binding. No heats of binding were detected with ITC, a method that is poor at detecting weak binding (mM  $K_d$ s), consistent with weak inhibition.

Next, separate  $^1\text{H}$  STD NMR spectra of **1** and **3** were recorded in the presence of MBP-MshC and the soluble MBP tag as control to determine the epitopes used for binding to *M. tuberculosis* MshC.<sup>18,19</sup> STD NMR is an especially powerful technique because it can be used to assign binding epitopes of ligands that bind their macromolecular receptors over a large spectrum of affinities ranging from high mM to sub-nM.<sup>17</sup> The  $^1\text{H}$  STD spectra of CysSA and AspSA in the presence of MBP-MshC are shown in Figure 4. Comparison of signals in the two STD spectra with those of the corresponding reference spectrum showed adenine H-2 ( $\delta$  8.15) to be most strongly enhanced for both analogs. (No binding was observed for either CysSA or AspSA to the soluble MBP tag.)



**Figure 4.** Binding of CysSA and AspSA to MshC by STD NMR. (A) Chemical structure of CysSA and AspSA with relative enhancements (%) shown in black and blue, respectively. STD NMR spectra<sup>19,20</sup> of (B) CysSA and (C) AspSA in the presence of MshC with reference and difference spectra colored black and red, respectively. Artefacts due to water suppression or impurities are marked with asterisks. Spectra were recorded as described in detail in Ref. 19. Weaker binding of AspSA to MshC relative to CysSA is apparent from the respective signal-to-noise (S/N) ratios of 20 and 90 in the difference spectra. S/N ratios for the CysSA and AspSA reference spectra are 170. Spectra were normalized to H-2. (D) Close up of CysSA in complex with *M. smegmatis* MshC with hydrogens in closest contact with the protein labeled.

Thus integrated peaks in the difference spectra were normalized to H-2 and the relative enhancements for CysSA and AspSA compared. As seen in Figure 4A, comparable enhancements for the purine and ribose moieties of **1** and **3** were observed with H-2 and H-1' showing the strongest enhancements. In contrast, differences in enhancements within the amino acid moiety are apparent. Relative enhancements for H-2'' of CysSA ( $\delta$  3.91) and AspSA ( $\delta$  3.80) are 21% and 12%, respectively. The H-3'' ( $\delta$  2.94) proton of **1** showed a 10% relative enhancement while no effect could be observed for H-3'' ( $\delta$  2.65) of **3**.

These data are consistent with interactions observed in the crystal structure of *M. smegmatis* MshC<sup>9</sup> in complex with CysSA where the purine H-2 is directed deep in the binding pocket, followed by H-8 and H-1' that are positioned further from the surface of the protein (Fig. 4D). In addition, these results indicate that although the adenosine portion of CysSA and AspSA, and presumably other adenosyl-containing compounds, can bind MshC through the adenosyl portion of the binding site, the discrimination observed for this enzyme occurs at the amino acid unit. Thus, although the GlcN-Ins acceptor used by MshC differs significantly in structure from the tRNA substrates of tRNA synthetases, the Cys-AMP binding domains exhibit comparable selectivity. A comparison of aminoacyl tRNA synthetases co-crystallized with aminoacyl adenosyl analogs showed that the adenosine and ribose moieties adopt similar orientations in the active site of most class I and II aa-tRNAs,<sup>20</sup> with H-2 and H-1' in close proximity to the protein, consistent with our STD data. In terms of MshC inhibitor design, our results suggest utility for adenosine analogs and/or small molecule thiolates, perhaps in the form of prodrugs, as MshC inhibitors.

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## Supplementary data

Supplementary data (NOESY spectra) associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2011.02.042](https://doi.org/10.1016/j.bmcl.2011.02.042).

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- NMR samples were prepared in Shigemi tubes containing 300  $\mu$ L of 20  $\mu$ M MBP-MshC (or MBP tag) and 900  $\mu$ M CysSA or AspSA in NMR buffer (20 mM NaCl, 20 mM phosphate, pH 6.5 in 99.9% D<sub>2</sub>O). The NMR buffer for CysSA also contained 2 mM dithiothreitol-*d*<sub>10</sub>. Detailed descriptions of the experimental NMR parameters used can be found in Ref. 18.
- Protein Data Bank identifiers for representative aminoacyl tRNA synthetases in complex with cognate aminoacyl adenosyl analogs include 2VOC (LeuRS/LeuSA), 2CT8 (MetRS/MetSA), 1N78 (GluRS/GluSA), 1JZQ (IleRS/IleSA), 1IVS (ValRS/ValSA), 2ZUE (ArgRS/ArgSA), 3CMQ (PheRS/PheSA) and 1FYF (ThrRS/SerSA).