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Mechanism-Based Inhibition of the *Mycobacterium tuberculosis* Branched-Chain Aminotransferase by D- and L-Cycloserine

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Abstract

The branched-chain aminotransferase is a pyridoxal 5'-phosphate (PLP)-dependent enzyme responsible for the final step in the biosynthesis of all three branched-chain amino acids, L-leucine, L-isoleucine, and L-valine, in bacteria. We have investigated the mechanism of inactivation of the branched-chain aminotransferase from *Mycobacterium tuberculosis* (*MtIvE*) by D- and L-cycloserine. D-Cycloserine is currently used only in the treatment of multidrug-resistant tuberculosis. Our results show a time- and concentration-dependent inactivation of *MtIvE* by both isomers, with L-cycloserine being a 40-fold better inhibitor of the enzyme. Minimum inhibitory concentration (MIC) studies revealed that L-cycloserine is a 10-fold better inhibitor of *Mycobacterium tuberculosis* growth than D-cycloserine. In addition, we have crystallized the *MtIvE*-D-cycloserine inhibited enzyme, determining the structure to 1.7 Å. The structure of the covalent D-cycloserine-PMP adduct bound to *MtIvE* reveals that the D-cycloserine ring is planar and aromatic, as previously observed for other enzyme systems. Mass spectrometry reveals that both the D-cycloserine- and L-cycloserine-PMP complexes have the same mass, and are likely to be the same aromatized, isoxazole product. However, the kinetics of formation of the *MtIvE* D-cycloserine-PMP and *MtIvE* L-cycloserine-PMP adducts are quite different. While the kinetics of the formation of the *MtIvE* D-cycloserine-PMP complex can be fit to a single exponential, the formation of the *MtIvE* L-cycloserine-PMP complex occurs in two steps. We propose a chemical mechanism for the inactivation of D- and L-cycloserine which suggests a stereochemically determined structural role for the differing kinetics of inactivation. These results demonstrate that the mechanism of action of D-cycloserine's activity against *M. tuberculosis* may be more complicated than previously thought and that D-cycloserine may compromise the *in vivo* activity of multiple PLP-dependent enzymes, including *MtIvE*.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.7b00142. Comparison of the minimal inhibitory concentration between DCS and LCS; ESI-MS analysis; Sequence alignment charts; Superimposition of structural diagrams (PDF)

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Notes

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The most recent report released by the World Health Organization (WHO) disclosed that tuberculosis (TB) is now the leading cause of human mortality by an infectious disease.¹ The causative agent of TB, *Mycobacterium tuberculosis*, resulted in approximately 1.5 million deaths in 2015 and is a major source of morbidity on HIV-positive patients.¹ The length of treatment (6–24 months) and the difficulties accessing effective drugs in developing countries, combined with the emergence of antibiotic resistant strains, are major challenges facing the fight against TB.² Therefore, a more thorough knowledge of the resistance mechanisms employed by *M. tuberculosis* against common antitubercular drugs, as well as the validation of new targets for the development of new drugs, is an urgent and unmet global health need.

D-Cycloserine (DCS) is an orally available, broad-spectrum antibacterial that was isolated in 1955 from a *Streptomyces* species.³ It was shown in 1960 to inhibit both the *Staphylococcus aureus* alanine racemase (AR) and D-ala-D-ala ligase (DAL), enzymes involved in the synthesis of the peptidoglycan component of bacterial cell walls, and was the first antibiotic whose mechanism of action was shown to be due to enzyme inhibition.⁴ DCS has been shown to be an irreversible inhibitor of bacterial D-amino acid transaminase,⁵ and the structure of the covalent pyridoxal 5'-phosphate (PLP)-DCS complex has been determined.⁶ The structure revealed that the DCS ring was intact and aromatic. This finding was also confirmed for the inactivation of γ -aminobutyric (GABA) aminotransferase by L-cycloserine (LCS).⁷ Structures of the DCS- and LCS-inhibited forms of dialkylglycine decarboxylase⁸ and of the LCS-inhibited forms of methionine γ -lyase⁹ have also been reported.

DCS is presently used almost exclusively as a second-line drug to treat multidrug-resistant TB (MDR-TB),^{10,11} due to its toxicity related to its activity as a mammalian neuronal NMDA receptor agonist and an increase in the levels of inhibitory neurotransmitters.^{12,13} The targets of DCS in *M. tuberculosis* were proposed to be similar to those in *S. aureus*; the PLP-dependent AR and DAL.¹¹ Early studies using genetic selection revealed that resistance to DCS was the result of mutations in the alanine-glycine-D-serine permease and the D-ala-D-ala ligase.¹⁴ Overexpression of the gene encoding alanine racemase was shown to confer resistance to DCS in *Mycobacterium smegmatis*.¹⁵ DCS was subsequently shown to be a slow, tight-binding inhibitor of the *M. tuberculosis* D-ala-D-ala ligase.¹¹ Two reports of

metabolomic profiling of DCS-treated mycobacterial cell extracts both concluded that D-alanine ligase was the primary, and lethal, target of DCS.^{16,17} A recent study demonstrated that clinical strains with low-level resistance to DCS mapped to mutations in the *ald* gene, encoding alanine dehydrogenase.¹⁸

Branched-chain amino acids are biosynthesized in *M. tuberculosis* in a manner similar to other eubacteria and by enzymes that have all been annotated in the genome.¹⁹ We have previously reported the three-dimensional structure of the *ilvE*-encoded (Rv2210c) branched-chain aminotransferase (BCAT) from *M. tuberculosis* (*MtIlvE*).²⁰ The PLP-dependent enzyme was crystallized in complex with pyridoxamine 5'-phosphate (PMP) and is a homodimer. *MtIlvE* catalyzes the L-glutamate-dependent amination of α -ketoisocaproate, α -keto-methyl-oxopentanoic acid, and α -ketoisovalerate to generate the three branched-chain amino acids (BCAAs) L-leucine, L-isoleucine, and L-valine.²¹ The enzyme is essential for growth under multiple growth conditions.^{22,23} Carbonyl reagents such as *O*-alkylhydroxylamines were inhibitors of the *M. tuberculosis* enzyme, the best exhibiting a K_i value versus L-leucine of 21 μ M and an MIC value against *M. tuberculosis* of 78 μ M.²⁴ The human BCAAT isozymes (cytosolic [*hBCATc*] and mitochondrial [*hBCATm*]; 58% sequence identity) are differentially inhibited by gabapentin.²⁵ *MtIlvE* shares approximately 34% sequence identity with *hBCATm* but is not inhibited by gabapentin.²⁰

In the present study, we examined the ability of DCS and LCS to inhibit *MtIlvE*. Our results show that both isomers inhibit the enzyme in a time- and concentration-dependent manner. However, LCS binds more tightly, and inhibits more rapidly, than does DCS and is a better inhibitor of mycobacterial growth. We crystallized the DCS-inhibited enzyme, and solved the structure to 1.7 Å. The structure of the enzyme-bound PMP-DCS adduct reveals that the DCS ring is intact and aromatic, as previously observed. Using absorbance spectra and mass spectrometry, we identified the *MtIlvE* LCS-PMP final complex as identical to that of the *MtIlvE* DCS-PMP complex. Based on our findings, we propose a mechanism of inhibition of *MtIlvE* by each of the cycloserine isomers based on stereochemical, kinetic, and structural evidence.

RESULTS AND DISCUSSION

Inactivation of *MtIlvE* by DCS and LCS

Mechanism-based inhibitors are a class of substrate analogs that lead to irreversible inhibition. Enzyme inactivation is caused by an initial chemical step identical to that normally occurring in catalysis followed by subsequent steps resulting in the formation of a stable dead-end complex, creating an irreversible protein-inhibitor complex.^{26,27} The highly conserved catalytic mechanism of PLP-dependent enzymes makes them particularly amenable to mechanism-based inhibitor design and enzyme inactivation.^{6,26–30} Due to their involvement in so many essential biological processes, a number of PLP-dependent enzymes have been targeted as potential drug targets.^{3,11–13}

The preincubation of *MtIlvE* with different concentrations of either DCS or LCS led to a decrease of enzymatic activity over time (Figure 1). The semilogarithmic plots of the

remaining activity as a function of time of inactivation in the presence of different concentrations of DCS and LCS indicates that the inactivation follows pseudo-first-order kinetics. Kitz and Wilson replots (insets, Figure 1) are linear for both DCS and LCS, suggesting that at least one molar equivalent of inhibitor binds to one molecule of enzyme for inactivation.³¹ In the case of the DCS-inhibited enzyme, the Kitz and Wilson replot suggests the formation of a low affinity inhibitor–enzyme complex which intercepts at the origin.^{29,32} This prevents an accurate estimation of K_I and k_{inact} values when using a linear regression method.^{29,32} No activity is recovered from the inactivated enzyme after several hours of dialysis, arguing that the inactivation is irreversible (data not shown). In comparison to DCS, LCS is a more efficient inhibitor of *MtlvE*. The K_I and k_{inact} values observed for LCS are 88 μM and $4.5 \times 10^{-4} \text{ s}^{-1}$, respectively. The stereospecificity displayed by *MtlvE*, which is an L-amino acid aminotransferase, explains the more rapid inactivation by LCS with the second-order rate constant of inactivation $k_{\text{inact}}/K_I_{\text{LCS}} = 7.32 \text{ M}^{-1} \text{ s}^{-1}$ being greater than $k_{\text{inact}}/K_I_{\text{DCS}} = 0.12 \text{ M}^{-1} \text{ s}^{-1}$. LCS is also a significantly better inhibitor than DCS of the PLP-dependent serine palmitoyl-transferase from *Sphingomonas paucimobili*, an enzyme that catalyzes the condensation of L-serine with palmitoyl-CoA in the biosynthetic pathway of sphingolipids.²⁹ LCS has been exploited in several model systems,^{8,33} including seizure models, due to its ability to increase intracerebral GABA levels.³⁴

We tested other PLP-dependent enzyme inactivators including propargylglycine, an inhibitor of cystathionine γ -synthase;²⁸ gabaculine, an inhibitor of GABA aminotransferase and ornithine decarboxylase;²⁷ and difluoromethylornithine (DFMO), the inhibitor of ornithine decarboxylase.³⁵ The preincubation of *MtlvE* with 10 mM of each of these inhibitors for over 1 h did not lead to any decrease in the activity of *MtlvE* (data not shown). It has been previously shown that gabapentin, a potent inhibitor of the *hBCATc*, has no effect on the activity of *MtlvE*.²⁴ Given our results, it is interesting that neither human BCAT isozyme is inhibited by DCS.³⁶ This clearly demonstrates that despite the sequence similarities shared among *MtlvE* and the human BCATs, especially *hBCATm*, the *M. tuberculosis* enzyme has the potential for selective inhibition.

Determination of MIC Values for LCS and DCS

In order to compare the inhibitory effects of LCS and DCS on *in vivo* growth of *M. tuberculosis*, growth inhibition experiments were carried out using an auxotrophic (panCD; requires pantothenate for growth) strain, *mc*²6230, derived from *M. tuberculosis* H37Rv. The MIC value of each compound was established as the lowest concentration of LCS or DCS that fully inhibits the growth of the bacteria after 15 days. DCS displayed a MIC value of 2.3 $\mu\text{g}/\text{mL}$ using this strain (Table S1). Previous studies on wild-type *M. tuberculosis* found MIC values for DCS equal to 15 $\mu\text{g}/\text{mL}$,¹⁸ while MDR-TB clinical isolates exhibited MIC values ranging from 32 to 64 $\mu\text{g}/\text{mL}$.³⁷ In general, laboratory strains are more susceptible to antibiotics than clinically isolated strains, such as MDR-TB strains. Similar experiments revealed that LCS exhibits a ~10-fold better inhibitory growth effect than DCS, with a MIC value of 0.3 $\mu\text{g}/\text{mL}$ (Table S1). Previous studies reported an increase in the MIC values in the presence of added L- and D-alanine,¹⁷ and we observed similar increases with the pantothenate auxotroph. The addition of either L-isoleucine, L-leucine, or L-valine, or all three, had no effect on the measured MIC values for either DCS or LCS (Table S1).

Electrospray Ionization–Mass Spectrometric Analysis of MtlvE DCS and LCS Complexes

To assess the identities of the final *MtlvE* complexes formed with LCS and DCS, we performed liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). The purified enzyme in the PLP form served as a control. The PLP released from the active enzyme yielded a peak at m/z 248, corresponding to the PLP cofactor, and a peak at m/z 150, corresponding to the loss of the phosphate group from the cofactor $[M + H - 98]^+$ (Figure S1).

The overnight incubation of *MtlvE* with 5 mM DCS or LCS leads to the complete inactivation of the protein. The covalent cycloserine-PMP complexes were then examined by LC-ESI-MS and confirmed by MS/MS. A peak at m/z 332 was observed for complexes generated by incubation with both DCS and LCS (Figure 2), corresponding to the predicted mass of the PMP-isoxazole covalent complexes (m/z 332.06, Scheme 1). The fragmentation data (MS/MS) for the DCS and LCS inhibited complexes were identical to each other (Figure S2) and similar to those reported previously for the LCS-inhibited γ -aminotransferase.⁷ Both DCS and LCS–cofactor complexes are fragmented in a way that makes it impossible to distinguish between the tautomeric PMP-isoxazole, internal aldimine, or ketimine forms of the adduct. However, previous reports of the concerted 1,3-prototropic shift mechanism of *MtlvE*²¹ and the structural studies presented below suggest the formation of a stable, aromatic PMP-isoxazole adduct.

UV–vis Analysis of MtlvE Inactivated by DCS and LCS

Enzymes containing the PLP cofactor can be kinetically analyzed using UV–vis spectrophotometry, since the absorbance is dependent on the chemical nature of the cofactor (PLP, PMP or quinonoid). PLP, bound as the internal aldimine to Lys204 in *MtlvE*, displays a characteristic UV–vis spectra with a visible absorbance maximum at 415 nm.²¹ The time course of the UV–vis spectral changes accompanying the transamination reaction catalyzed by *MtlvE* starts with a PLP Schiff-base or internal aldimine ($\lambda_{\max} \approx 415$ nm) that is converted to the next stable enzyme form, PMP ($\lambda_{\max} \approx 330$ nm). A quinonoid intermediate often observed at 490 nm is absent in the reaction catalyzed by *MtlvE* due to its concerted 1,3-prototropic shift mechanism.²¹ In the presence of 1 mM DCS, a similar pattern is observed (Figure 3A). In 60 min, the conversion of the 415 nm peak representing the internal aldimine is reduced and a 330 nm peak indicative of a PMP derivative appears. There is a very clear isosbestic point observed at *ca.* 350 nm, and a plot of the A_{420} vs time can be fit to a first-order decrease of $[E-PLP]$. This result suggests that the *MtlvE*-bound cofactor forms a complex with DCS to generate a covalent DCS-PMP complex ($\lambda_{\max} = 330$ nm) where the 2-oxazolidine ring is intact and has aromatized, as reported for other enzyme systems.^{15,38}

Significant differences in the time course of inhibition of *MtlvE* by LCS are observed spectrophotometrically in the UV–vis region (Figure 3B). The addition of 0.5 mM LCS to *MtlvE* led to a rapid (~10 min) but modest decrease of the internal aldimine peak (415 nm) with a corresponding modest increase at approximately 370 nm and *without* a corresponding increase at 330 nm. An isosbestic point at approximately 380 nm was reported for the time course of LCS inhibition of serine palmitoyltransferase from *S. paucimobili*.²⁹ These

spectral changes were proposed to be due to the formation of an oxime intermediate, and a similar spectrum was observed for GABA aminotransferase inhibited by hydroxylamine.³³ However, this transient intermediate is slowly replaced at longer times (>20 min) by spectral features equivalent to those observed during reaction of the enzyme with DCS. At these longer times of reaction, the spectrophotometric changes are interpretable as the conversion of the PLP form of the enzyme into a PMP-LCS adduct identical to that observed for DCS and exhibiting a clear isosbestic point indicative of this conversion. If we again plot the A_{420} as a function of time, it is clear that a first-order decrease in the absorbance at times less than 8–10 min is not observed, while a two-exponential fit conforms to all the data. The kinetic and stereochemical interpretation of these differences will be discussed below.

Overall Structure of MtlvE-PLP-DCS

MtlvE incubated with DCS until complete inactivation of the enzyme was achieved was crystallized, and the structure of *MtlvE*-PMP-DCS was solved to 1.7 Å. Data collection and refinement statistics are summarized in Table 1. The complex crystallized with two monomers in the asymmetric unit, covalently linked by an interchain disulfide bond. Tremblay et al. previously solved the *MtlvE*-PMP structure and showed that *MtlvE* forms a dimer in solution.²⁰ Extremely low r.m.s. displacement values for the C α atom positions (0.18 Å) is observed when the *MtlvE*-PMP structure is superimposed onto the *MtlvE*-PMP-DCS structure, indicating both structures are extremely similar.

The *MtlvE* structure displays the characteristic type IV fold of PLP-dependent enzymes,²⁰ with each monomer consisting of a small and a large domain that define the two active sites at the domain interfaces. The first domain includes both N- and C-terminal residues (residues 34–173 and 362–368) forming three α -helices and eight β -strands while the second domain is composed of residues 182 to 362, displaying nine β -strands and three α -helices (Figure 4). A rigid α -helix between residues E340 and R354 connects the two domains. A flexible loop (residues 173–182) should also link both domains, but no electron density was observed and the loop was not built into the *MtlvE*-PMP-DCS model.

As previously observed, the N-terminal portion of the protein is highly disordered and electron density is only observable from H35 in monomer A and Y34 in monomer B in the *MtlvE*-PMP-DCS structure. Additionally, a loop consisting of residues G174 to I181, which connect the two domains of *MtlvE*, is poorly resolved in this structure. This linker loop is located near the active site and is highly flexible due to the presence of two glycine residues (G179-G180). This linker loop has been shown to play a significant role in the entrance and exit of the substrates/products.^{39,40} In contrast to aspartate aminotransferase, that exhibits a conformational change involving the small domain closing onto the active site upon binding of the substrate,^{41–44} only the linker loop between the small and large domains moves to close the active site in BCAT enzymes.^{39,40,45}

Cys196 Disulfide Bond

In the *MtlvE*-PMP structure, Tremblay et al. observed cysteine residues (Cys196) that were located at the homodimer interface 3.6 Å away from its identical symmetry-mate residue.²⁰ There was no evidence in that structure that the two cysteine residues form a disulfide bond,

but based on their separation, it was clear that they could under oxidative conditions. In contrast, the *MdlvE*-PMP-DCS structure exhibits clear 2Fo-Fc electron density supporting the presence of a disulfide linkage between the Cys196 residues from each monomer. The distance between the S γ atoms in the intersubunit disulfide bond is 2.05 Å, an appropriate bond length distance. The formation of an intersubunit disulfide bond had previously been hypothesized to increase the stability of the *MdlvE* dimer under the oxidative conditions observed within the macrophages.²⁰ Cysteine residues have been shown to play a significant role in the regulation of catalytic activity of *hBCATm*.^{45–47} A C₃₁₅XXC₃₁₈ motif is conserved among all mammalian BCAT enzymes and the formation of a C315–C318 disulfide bond leads to a loss in activity of *hBCATm*. The cysteine residues involved in the disulfide bridge in the *MdlvE*-PMP-DCS structure are structurally located in a different region, and C196 is not a conserved residue among other BCAT enzymes (Figure S3).

Enzyme Interaction with PMP-Cycloserine Adduct

Clear 2Fo-Fc and Fo-Fc electron density, corresponding to a PMP-D-cycloserine adduct, was observed in the active site of *MdlvE*-PMP-DCS. Only an intact ring form of cycloserine could account for the Fo-Fc electron density observed. This result is in agreement with the mass spectrometry data. The adduct appears tightly bound within the *MdlvE* active site as supported by the low B-factors observed (less than 25 Å²). The pyridoxamine moiety of the PMP-cycloserine derivative lies in the same conformation as PMP in the *MdlvE*-PMP model (Figure 5A). The cycloserine ring replaces a water molecule (W423) in the previously solved apo structure. Similar interactions to the ones previously observed in the *MdlvE*-PMP active site are observed in this new structure (Figure 5B,C). The N1 atom of the pyridine moiety interacts with E240 via hydrogen bonding and the ring hydroxyl group (O3) is forming a hydrogen bond with the phenolic moiety of Y209, as well as a water molecule (W333). The oxygen atoms of the phosphate moiety form multiple polar interactions with several residue side chains (R101, T272, and T314), backbone nitrogen groups (I271 and T314), and water molecules W4 and W41 (Figure 5B,C). The exocyclic N atom of cycloserine lies close (2.8 Å) to K204 (Figure 5C).

The cycloserine ring appears tilted compared to the plane of the pyridine ring. The geometry indicates an sp³ hybridization of the C4A atom, confirming the identity of the PMP form of the cofactor. Additionally, the five-membered ring is planar and is consistent with the presence of an aromatic, isoxazole ring. The cycloserine portion of the adduct makes polar interactions with residues within the active site. N2 forms a hydrogen bond (2.6 Å) with the phenolic group of Y144. The C3–O atom (presumably as the alkoxide) of the cycloserine isoxazole ring interacts with the catalytic residue K204 (2.9 Å), as well as two water molecules (W333 and W634). This structure is similar to the previously reported structure of D-amino acid aminotransferase crystallized after reaction with DCS.⁴⁸ Superposition of the two structures reveals a low overall r.m.s. displacement (1.8 Å), and a detailed comparison of the active site reveals a large number of conserved interaction between the PMP-DCS complex and side chain residues (Figure S4).

Mechanistic Analysis of the Inhibition of *MtlvE* by DCS and LCS

The very different kinetic behavior of the two stereoisomers of cycloserine was somewhat unexpected considering the identity of the final products of the pyridoxal adducts with the two compounds. LCS was a significantly more efficient inhibitor of *MtlvE* than DCS and also more effective in inhibiting the growth of *M. tuberculosis*. The high-resolution structure of *MtlvE*-PMP-DCS allows us to propose a detailed chemical mechanism for the inhibition by the two isomers and speculate about the relative kinetics of the steps leading up to the final aromatized products. While both LCS and DCS bind as analogs of the natural amino donor, L-glutamate, LCS is likely to bind and assume a catalytically appropriate orientation more rapidly. As shown in Scheme 1, the N2–C3 *N*-hydroxyamide function of the 2-oxazolidine ring is most likely bound as the ionized C3 hydroxyl anion, mimicking the carboxyl group of an L-amino acid. The stereochemistry of LCS at the equivalent of the $C\alpha$ center, coupled with the position of the K204 side chain that functions to remove the $C\alpha$ proton below the plane of the PLP ring, and the requirement that the $C\alpha$ -H bond be oriented perpendicular to the plane of the conjugated external aldimine for maximal stereoelectronic overlap allows us to draw the precatalytic PLP-LCS complex as shown (Figure S5). In this orientation, the C3 hydroxyl anion can interact electrostatically with R101 and is on the “C5-phosphate” side of the PLP ring and the C4- α H is within 2.7 Å of K204 in a model of the precatalytic complex. Removal of the α H is expected to be fast from this complex, generating the ketimine intermediate, in which the orientation of the oxazolidine ring is now fixed due to the restricted rotation about the cofactor–inhibitor imine linkage. These two steps, external aldimine formation followed by deprotonation to generate the ketimine, may be the rapid steps that are reported on in the early (<10 min) spectral traces of inactivation by LCS (Figure 3B). This argues that the final step, C5–H abstraction to generate the aromatized and stable 2-isoxazole adduct, is slow from the LCS-generated ketimine.

In contrast, the time course of the UV–vis changes accompanying DCS inhibition (Figure 3A) of *MtlvE* suggest that the opposite stereochemistry is associated with changes in the relative rates of these same steps discussed above. DCS may bind initially in the “LCS orientation” with the C3 hydroxyl anion on the “C5-phosphate” side of the pyridoxal ring, but must ultimately rotate to allow for C4- α H removal by K204, placing the C3 hydroxyl anion on the “C3-hydroxyl” side of the pyridoxal ring. This is likely to make the formation of the precatalytic external aldimine conformation slower than with LCS. Proton abstraction, accompanied by a 1,3 prototropic shift, generates the ketimine, which differs from the ketimine generated from LCS in the orientation of the oxazole ring and the positioning of the C5 protons. Proton abstraction from C5 of the oxazole ring leads to the final aromatized PMP–isoxazole covalent complex. Since the stereochemical differences are lost as a result of $C\alpha$ -H abstraction, as are any rotational restrictions in the PMP-LCS and PMP-DCS adducts, it is unclear whether the crystallographically observed orientation of the ring for the PMP-DCS adduct (Figure 5B) is also not adopted by the PMP-LCS adduct after ring aromatization.

CONCLUSION

Branched-chain aminotransferases are responsible for essential processes in mammalian catabolism and eubacterial biosynthesis. Significant efforts have been made toward the development of lead compounds capable of inhibiting the activity of the *hBCATm*, an enzyme that has been implicated in human obesity.⁴⁹ In addition to its role in the biosynthesis of L-isoleucine, L-leucine, and L-valine, *MtlvE* is implicated in the biosynthesis of L-methionine and catabolism of aromatic amino acids,^{21,24} suggesting that *MtlvE* is a suitable therapeutic target.

While the essential, and lethal, targets of DCS in *M. tuberculosis* have been demonstrated,^{11,18} our data suggest that the branched chain amino acid aminotransferase, *MtlvE*, is a *bona fide* target of both DCS and LCS. DCS inhibits several PLP-dependent enzymes in multiple organisms,^{6,8,12,29,50} including the *M. tuberculosis* alanine racemase and D-alanine ligase involved in peptidoglycan biosynthesis. The rate of development of resistance to DCS is very low compared to other antitubercular drugs,^{14,15,18} which has been attributed to the overexpression of DAL and AR.^{10,14,15}

The unusually robust growth-inhibiting activity of LCS compared to DCS suggests that this compound may have a different, and broader, range of inhibitable targets, including *MtlvE*. Of the known DCS targets, the effects of LCS are likely due to the inhibition of AR since D-alanine-D-alanine ligase is not inhibited by LCS.¹⁷ Our MIC data show that both D- and L-alanine substantially increase the MIC value for LCS supporting the conclusion that alanine racemase is the primary target under these conditions. While our biochemical and structural data clearly support *MtlvE* as being inhibitable by both DCS and LCS, under the conditions used, a similar “protective” effect of added branched-chain amino acids is not observed. Instead, a pleiotropic effect on multiple, essential PLP-dependent enzymes, including *MtlvE* may be the reason for the robust activity of LCS against *M. tuberculosis*. While the current drug design principle of “one drug-one target” has been effective, perhaps “death by a thousand cuts” might be equally effective as an antibacterial therapeutic paradigm.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical or reagent grade and did not undergo further purification. DCS and LCS were purchased from Sigma-Aldrich.

Expression and Purification of *MtlvE*

MtlvE (encoded by the recombinant pET28a(+):*tlvE* plasmid) was expressed and purified using the protocol previously reported.²⁰ The protein was dialyzed against 50 mM Tris HCl, pH 8.0 (Buffer A). Glycerol (50% v/v) was added to the protein sample for long-term storage at -20 °C. The protein concentration was determined by absorbance spectroscopy at a wavelength of 280 nm using the theoretical extinction coefficient ($\epsilon_{280} = 56\,380\text{ M}^{-1}\text{ cm}^{-1}$) calculated with the ProtParam function on the ExPASy server.⁵¹

Activity Assay and Inactivation by D- and L-Cycloserine

MtlvE activity was measured continuously using a coupled spectrophotometric assay including bovine liver glutamate dehydrogenase (GDH; Sigma), excess reduced nicotinamide adenine dinucleotide (NADH) and ammonia as previously described.²¹ The activity was monitored by the decrease in absorbance at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) on a UV-2450 Shimadzu spectrophotometer.

Inactivation assays were performed by incubating 40 nM of *MtlvE*-PLP form with DCS (1 mM, 2 mM, 3 mM, and 4 mM) or LCS (25 μM , 50 μM , 75 μM , and 100 μM). The remaining activity was measured at different time points (0, 10, 20, 40, and 60 min) after addition of the assay mixture. The latter contained 15 mM L-glutamate, 2 mM α -ketoisocaproate, 40 nM PLP, 1 mM ammonium chloride, 160 μM NADH, and 8 units of GDH in a total reaction volume of 1 mL. The reaction was initiated by the addition of aliquots from the inactivation mixture. All measurements were performed in duplicate at 25 °C using buffer A (50 mM Tris HCl, pH 8.0).

A percentage of the total activity in the absence of inhibitor or time zero was calculated. Kitz and Wilson plots of $\ln$ % remaining activity versus time were linear.⁵² All data points are from an average of duplicate or triplicate measurements, and all data were analyzed by nonlinear regression using the SigmaPlot software (Systat Software, Inc.) and fitting to eq 1⁵³

$$\ln \left(\frac{E(t)}{E(0)} \right) = \frac{-k_{\text{inact}} \times t}{\left(1 + \frac{K_I}{[I]} \right)} = -k_{\text{app}} \times t \quad (1)$$

where $E(0)$ represents the activity prior to addition of the inhibitor, $E(t)$ is the activity after addition of either DCS or LCS at time = t , k_{app} is the inactivation rate at any inhibitor concentration = $[I]$, K_I is an estimate of inhibitor binding affinity, and k_{inact} is the first-order inactivation rate constant. By plotting $1/k_{\text{app}}$ versus $1/[I]$, a straight line with an intercept of $1/k_{\text{inact}}$ and slope of K_I/k_{inact} was obtained.

UV-vis Spectroscopy of *MtlvE* Inhibition by DCS and LCS

MtlvE was incubated with α -ketoisocaproate for 60 min to ensure that all active sites of the enzyme were converted into the internal aldimine (Schiff-base) form. The protein was dialyzed against buffer A to remove excess PLP and leucine formed during the incubation. The concentration of PLP bound to the enzyme was determined using absorbance spectroscopy at a wavelength of 415 nm ($\epsilon_{415} = 4900 \text{ M}^{-1} \text{ cm}^{-1}$) as previously calculated.²¹ UV-vis time course experiments were carried out using 100 μM *MtlvE* and the spectrophotometer was blanked with buffer A. One milliliter quartz cuvettes were used, and spectra from 550 to 240 nm were recorded upon the addition of 1 mM DCS or 0.5 mM LCS at different times.

Mass Spectrometry

MilvE-PLP form (100 μ M) was incubated with 5 mM LCS or DCS overnight at RT in buffer A. An Amicon device (10K molecular weight cutoff) was used to remove the cycloserine excess by centrifugal filtration at 4 °C for 15 min at 4000 rpm. A negative control was carried out without addition of either drug. LC-ESI-MS was performed in order to confirm the molecular weight of the final complexes *MilvE*-DCS and -LCS. A ThermoFinnigan, LTQ Orbitrap Velos mass spectrometer was used at 15 000 resolution (at m/z 400) in positive ionization mode and scanned from 200 to 500 m/z . The mobile phase A was composed of 5% v/v acetonitrile/0.1% v/v formic acid, and the mobile phase B consisted of 100% v/v acetonitrile/0.1% v/v formic acid. We used an HPLC Cogent Diamond Hydride 150 mm $\times$ 2.1 mm column. Step gradient was as follows: 0 to 5 min at 2% phase B; 5 to 6 min 20% phase B; 6 to 8 min 40% phase B; 10 to 12 min 60% phase B; 12 min 80% phase B; 12 to 19 min 90% phase B; and held to 90% up to 30 min. Approximately 50 mL of sample without further processing was injected. After 4.5 min of desalting to waste, the tubing outlet from the column was connected to the mass spectrometer. Samples started being collected at 5 min. Elution time was 27 min.

Determination of MIC Value for DCS and LCS

In vivo growth inhibition studies were performed using a BSL2-safe derivative strain of *M. tuberculosis* H37Rv called mc²6230 (H37Rv panCD), which is auxotroph strain for pantothenate (pan⁻).⁵⁴ The auxotroph strain was maintained in Middlebrook 7H9 broth (Difco) supplemented with 10% v/v ADC (5% v/v bovine serum albumin [BSA], 2% v/v dextrose, 5% v/v catalase) 0.025% v/v tyloxapol, and 50 μ g/mL of pantothenate. For the rescue experiments, 1 mM of the following amino acids were individually added to the cultures: L-alanine, D-alanine, L-glutamate, L-isoleucine (I), L-leucine (L), L-valine (V), and ILV. Both DCS and LCS were dissolved in water to a stock concentration of 0.5 mg mL⁻¹ and sterilized by filtration. From logarithmically growing cultures, aliquots were transferred to supplemented 7H9 media (as described above) to an OD₆₀₀ = 0.01. Following standard 1.5-fold dilutions, dose-response assays were performed in triplicate using a 96-well plate (Corning Incorporated Costar) in 100 μ L volume with final concentrations ranging from 135 to 0.012 μ g/mL. Cultures without LCS or DCS served as positive controls and cultures with 100 μ g/mL kanamycin served as negative controls. After 15 days of incubation at 37 °C, 30 μ L of resazurin dye (filter sterilized, 0.2 mg mL⁻¹ concentration) was added to each well and incubated for 6 h under shaking and the plates were imaged. The MIC (minimum inhibitory concentration) was defined as the lowest concentration that completely inhibits the growth of mc²6230 at day 15, as indicated by resazurin dye staining.

Crystallization

MilvE (20 mM HEPES, pH 7.5) containing an N-terminal His₆-tag was concentrated to 6 mg mL⁻¹ using an Amicon Ultracentrifugal filter. The sample was incubated with 0.2 mM PLP and 10 mM DCS for 3 h at 25 °C. The inactivated protein was screened in a 1:1 ratio against the commercially available screen MCSG suite (Microlytic). The screening was performed utilizing the sitting-drop vapor diffusion technique in a 96-well plate format (Intelli-Plates 96, Art Robbins). Reservoir solutions and drops were dispensed using a

Crystal Gryphon robot (Art Robbins). The crystal plates were incubated at 20 °C. The screening yielded single crystals grown against a well solution containing 0.1 M Bis-Tris, pH 6.5, and 25% w/v polyethylene glycol 3350. The crystals were obtained after approximately six to eight weeks of incubation. The crystal was soaked with 25 mM D-cycloserine for 10 min prior to flash cooling in liquid nitrogen.

Data Collection and Structure Determination

Diffraction data were collected at the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at the Advance Photon Source Argonne National Laboratory (APS-ANL, IL) at 0.97931 Å wavelength radiation. Diffraction data were indexed, integrated, and scaled to 1.7 Å using the XDS program package.⁵⁵ Molecular replacement was carried out with the software Phaser-MR using PDB 3HT5 as the search model.^{20,56} Two molecules per asymmetric unit were observed. Using the solution obtained by molecular replacement, the refine tool available in the PHENIX suite was used to perform rigid body refinement simulated annealing, positional and B-factor refinements.⁵⁰ The model was manually corrected using COOT.⁵⁷ Clear 2Fo-Fc and Fo-Fc electron density maps corresponding to a PLP-cycloserine adduct were observed. The geometric restraints of the PLP-cycloserine adduct, represented by the three-letter code DCS, were determined using eLBOW (electronic Ligand Builder and Optimization Workbench) in PHENIX.⁵⁸ MOLPROBITY was used to assess the quality of the structure.⁵⁹ Two residues were observed in the disallowed region of the Ramachandran plot (V317 in each monomer).⁶⁰ The structure was deposited in the Protein Data Bank (PDB accession code 5U3F).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AR	alanine racemase
BCAAs	branched-chain amino acids
BCATs	branched-chain aminotransferases

DAL	D-alanine-D-alanine ligase
DCS	D-cycloserine
DFMO	difluoromethylornithine
GABA	γ -aminobutyric acid
GDH	glutamate dehydrogenase
LCS	L-cycloserine
MDR	multidrug-resistant
<i>MtIlvE</i>	branched-chain aminotransferase IlvE
NADH	nicotinamide adenine dinucleotide
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
TB	tuberculosis
XDR	extensively drug-resistant
WHO	World Health Organization

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Figure 1.

Mechanism-based inhibition of *MtlvE* by (A) DCS and (B) LCS. (A) $\ln$ % of remaining activity of *MtlvE* inhibited over time and increasing concentrations of DCS as follows: (♦) control, (▼) 1 mM, (▲) 2 mM, (■) 3 mM, and (●) 4 mM. (B) $\ln$ % of remaining activity of *MtlvE* inhibited over time and increasing concentrations of LCS as follows: (♦) control, (●) 25 μM , (■) 50 μM , (▲) 75 μM , and (▼) 100 μM . The insets represent the Kitz-Wilson replots and describe the $t_{1/2}$ life. The second-order rate constant k_{inact}/K_I is calculated as $1/\text{slope}$.

Figure 2. ESI-MS analysis of intact molecular weights of *MtlvE*-PLP inhibited overnight by (A) DCS and (B) LCS. The peak at m/z 332.06 represents, in both cases, the presence of a noncovalent protein inhibitor complex where the cycloserine ring is intact.

Figure 3.

UV-vis analysis of *MdlvE* inhibition by the cycloserine isomers over time. The upper blue lines on (A) and (B) represent *MdlvE*-PLP in the absence of inhibitor. All the other lines represent the spectra of *MdlvE* in the presence of (A) 1 mM DCS at 1, 6, 12, 15, 25, 35, 45, 60, 90, 120, 180, and 300 min, respectively. The inset in (A) represents the change in absorbance at $\lambda_{\text{max}} \approx 415$ nm over time. The data was fit to a single exponential equation; and (B) 0.5 mM LCS at 1, 6, 25, 45, 90, 120, 180, 240, 300, 360, 420, 600, 1050, and 1665 min, respectively. The two lower panels represent the change in absorbance at $\lambda_{\text{max}} \approx 415$ nm over time. The data on the left lower panel was fit to a single exponential equation. The right lower panel was fit to a double exponential equation. In both (A) and (B), the peak at $\lambda_{\text{max}} \approx 415$ nm (internal aldimine) is reduced over time, while the PMP ($\lambda_{\text{max}} \approx 330$ nm) peak is increased. The appearance of the PMP in the DCS inhibited enzyme happens approximately 5-fold faster than with LCS.

Figure 4.

Overall structure of *MtlvE* homodimer in complex with PMP-DCS. The two domains of *MtlvE* are colored in orange (residues 34–173 and 363–368) and green (residues 182–362), respectively. An α -helix (residues 340–354, colored in blue), as well as a flexible loop (residues 173–182, poorly resolved in this structure), connect the two domains. The PLP-cycloserine molecule is rendered as sticks colored by CPK, with carbon atoms in white. A red circle highlights the presence of a disulfide bond covalently linking the two monomers. Close-up view (inset) of the interchain disulfide bond observed in the *MtlvE*-PMP-DCS structure. The cysteines C196 are involved in the formation of a disulfide bond located at the homodimer interface. The electron density of a 2Fo-Fc map (blue) is shown contoured at 1 σ . The two residues are represented as sticks colored by CPK, with carbon and sulfur atoms colored in green and yellow, respectively.

Figure 5.

Active site of the *MtlvE*-PLP-DCS structure. (A) Superimposition of the *MtlvE*-PMP (orange) and *MtlvE*-PLP-DCS (green) structures. Residues interacting directly with either PMP or PLP-DCS adduct are representing as sticks colored by CPK whereas the water molecules are rendered as spheres. When two water molecule positions overlap, the first number corresponds to the *MtlvE*-PLP-DCS structure and the second to the *MtlvE*-PMP structure. W423 was replacing the cycloserine ring in the *MtlvE*-PMP structure. (B,C) The electron density of a Fo-Fc omit map (blue) is shown contoured at 3σ , the PLP-cycloserine molecule was omitted during map calculation. PLP-cycloserine is represented as sticks colored by CPK, with carbon atoms in yellow. The residues participating in hydrogen bonding (dashes) with PLP-cycloserine are rendered as sticks, while the water molecules

(W) interacting with the ligand are represented as spheres. The distances are given in angstroms.

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Scheme 1.

Proposed Aromatization Mechanism of Inactivation of MtlIvE by LCS (left) and DCS (right)

Table 1

Data Collection and Refinement Statistics

data collection	<i>MtIlvE</i> -PLP-cycloserine
PDB ID	
Space Group	$P2_12_12_1$
Unit Cell Dimensions	
a, b, c (Å)	91.9; 80.5; 81.3
α, β, γ (deg)	90.0; 90.0; 90.0
Resolution Range (Å)	50.0–1.70
Wavelength (Å)	0.97931
Redundancy (Highest shell)	3.8 (3.6)
R_{merge} (%) (Highest shell)	6.8 (54.2)
CC(1/2) (Highest shell)	99.8 (79.7)
$I/\sigma I$ (Highest shell)	12.5 (2.4)
Completeness (%) (Highest shell)	91.1 (98.1)
Total Reflections (Unique)	442066 (117641)
Refinement	
$R_{\text{work}}/R_{\text{free}}$	17.6/20.9
Number of non-hydrogen atoms	5655
Protein	4994
Water	617
Ligand	44
Average B-factors (Å ²)	24.0
Protein	23.1
Water	30.7
Ligand	19.4
Wilson B factor	19.5
r.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (deg)	1.206
Ramachandran plot	
Favored (%)	98.0
Outliers (%)	0.3