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# Metabolomics Reveal D-Alanine:D-Alanine Ligase As the Target of D-Cycloserine in *Mycobacterium tuberculosis*

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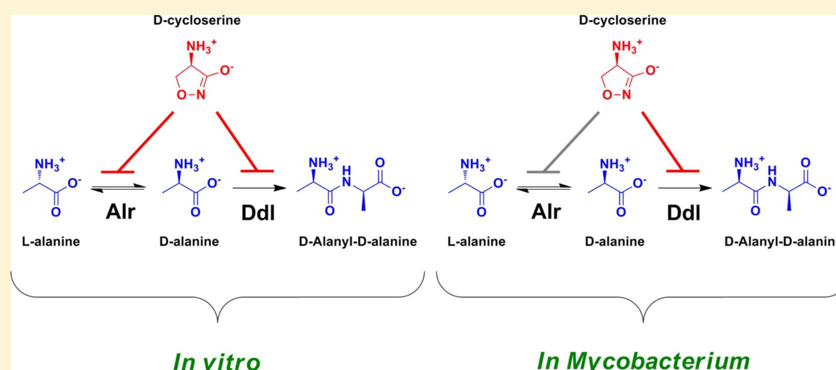
Supporting Information




**ABSTRACT:** Stable isotope-mass spectrometry (MS)-based metabolomic profiling is a powerful technique for following changes in specific metabolite pool sizes and metabolic flux under various experimental conditions in a test organism or cell type. Here, we use a metabolomics approach to interrogate the mechanism of antibiotic action of D-cycloserine (DCS), a second line antibiotic used in the treatment of multidrug resistant *Mycobacterium tuberculosis* infections. We use doubly labeled $^{13}C$ $\alpha$-carbon-$^{2}H$ L-alanine to allow tracking of both alanine racemase and D-alanine:D-alanine ligase activity in *M. tuberculosis* challenged with DCS and reveal that D-alanine:D-alanine ligase is more strongly inhibited than alanine racemase at equivalent DCS concentrations. We also shed light on mechanisms surrounding D-Ala-mediated antagonism of DCS growth inhibition and provide evidence for a postantibiotic effect for this drug. Our results illustrate the potential of metabolomics in cellular drug-target engagement studies and consequently have broad implications in future drug development and target validation ventures.

**KEYWORDS:** *Tuberculosis, peptidoglycan, mechanism of action, cycloserine, metabolomics*

Increasing global prevalence of drug resistance and disease incidence across many infectious diseases underscores an urgent requirement for novel, cost-effective therapeutics. Low success rates in standard target-based and whole-cell screening drug discovery ventures, however, suggest that new approaches are required to identify drug features important for potency and specificity.[1] While the advent of molecular genetics has been instrumental in defining individual antibiotic targets and resistance mechanisms, little is known about the pharmacodynamics and -kinetics of drugs within the target organism. Defining the global changes in cellular performance upon drug treatment is essential if activities are to be emulated in novel therapeutics. Metabolomics, the study of a cell's metabolic status in terms of metabolite pool sizes and pathway flux, is a powerful and emerging field that, through recent technological advances in mass spectrometry (MS) and nuclear magnetic resonance (NMR), allows high-resolution mapping of perturbations in cellular metabolism following drug treatment or introduction of a defined genetic lesion.[2] As the metabolome corresponds to the ultimate read-out of a cell's physical health, metabolomics represents a potent tool in the discovery of drug mechanism of action. In addition, direct interaction of the drug with its putative target (target engagement) can be monitored in several cases. In a similar fashion to target engagement studies by activity-based protein profiling,[3] stable isotope labeling coupled to metabolomic approaches can inform on in vivo enzyme inhibition.

D-Cycloserine (DCS, Scheme 1), an FDA-approved antibiotic for the treatment of multi- and extensively drug resistant tuberculosis, constitutes a drug with an ill-defined mode of action, but whose efficacy and low rates of clinical resistance indicate a valuable resource for future drug development.[4] Dose-limiting host-toxicity, however, principally in the form of neuronal NMDA-receptor partial agonism,[5] currently restricts usage, and therefore, improvements in target specificity would benefit next-generation DCS analogues. In vitro, DCS is a competitive inhibitor of two bacterial-specific enzymes involved in the D-Alanine pathway of peptidoglycan biosynthesis (Scheme 1): alanine racemase (Alr) and D-alanine:D-alanine ligase (Ddl).[6] While both enzymes are essential in almost all

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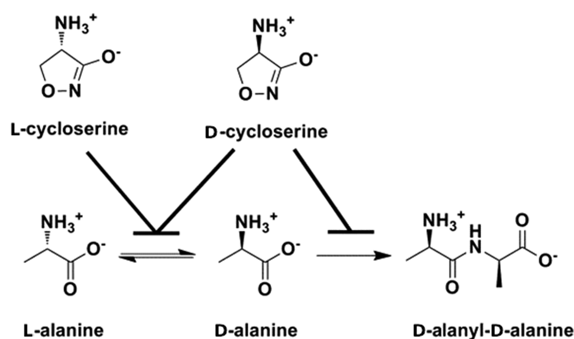
**Scheme 1. Bacterial D-Alanine Pathway and Proposed Molecular Targets of LCS and DCS**




bacterial species studied to date, including the primary clinical target *Mycobacterium tuberculosis*,[7] ambiguity still exists over the precise lethal target of DCS. While the argument in favor of Alr is largely based on the well-defined mechanism of irreversible inhibition by DCS,[8] mounting evidence suggests it is not the primary target in *Mycobacterium smegmatis*, a fast-growing organism often used as a surrogate of *M. tuberculosis*.[9,10] Also, studies with recombinant *M. tuberculosis* Ddl indicate that DCS inhibition is likely to take place at similar or lower concentrations required to attain inhibition of Alr.[11] The critical roles of alternative targets, particularly Ddl, in DCS antibiotic action have therefore yet to be elucidated, in particular in *M. tuberculosis*. Here, we use stable isotope LC-MS based metabolomics to define the relative roles of Alr and Ddl in the mechanism of action of DCS in *M. tuberculosis*.

Previous studies have demonstrated that exogenously added D-Ala, but not L-Ala, is able to rescue growth inhibition in DCS-challenged bacteria,[12,13] and we have reconfirmed these results using *M. tuberculosis* H37Rv (Figure 1a). We also show that growth inhibition by LCS (Scheme 1), the optical isomer of DCS that retains inhibitory activity against Alr but not against Ddl,[8,14] can be rescued by media supplementation with both D-Ala and L-Ala. These results can be interpreted as L-Ala out-competing LCS binding for Alr but being unable to antagonize DCS binding to Ddl, while D-Ala provides protection by out-competing DCS binding to Ddl or bypassing the requirement for Alr. The difference between D-Ala outcompeting binding to Ddl in the case of DCS and directly bypassing Alr in the case of LCS can be observed in the relative strength of rescue between the two compounds at similar D-Ala concentrations: 20 $\mu$M D-Ala doubles the MIC for DCS but quadruples that of LCS. Additionally, antibiotic activity of DCS synergizes with both LCS and $\beta$-chloro-D-alanine (BCDA), another unique Alr-inhibitor (Figure 2b).[8,15] In contrast, no synergistic activity is observed with the translation-inhibitor streptomycin. Such synergistic activity is characteristic of a blockage in consecutive steps of a single pathway[16,17] and supports a predominantly Ddl-inhibitory mode of action for DCS.

Using a validated filter-based technique coupled to high-resolution MS, we next studied the changes in intracellular pool sizes over time, following DCS treatment, of the main metabolites involved in the peptidoglycan D-alanine pathway. Growth in the presence of increasing concentrations of DCS up to 5× the MIC led to a rapid and dose-dependent depletion of the dipeptide D-Ala:D-Ala pool to near-zero values (Figure 2a). This is consistent with previous studies in alternative bacterial species[9,18] and supports peptidoglycan biosynthesis inhibition as the lethal effect of DCS. In accord, streptomycin at 5× MIC had no effect on D-Ala:D-Ala levels (Figure 2a, dashed dark gray line). However, D-Ala:D-Ala depletion is also induced by LCS (Figure 2a, dashed light gray line) and is therefore insufficient evidence by itself to define Ddl as the primary target of DCS. Relative pool sizes of L- and D-Ala were unable to be determined due to lack of chiral separation with the chromatography employed; however, comparison of differential effects on total Ala pool sizes between DCS and LCS treated samples (Figure 2b) revealed distinct mechanisms of action. While 1× MIC of LCS rapidly reduced the total Ala pool (Figure 2b gray dashed line), 0.25× and 1× MIC of DCS caused a transient increase before returning to preinhibited levels at later time points (Figure 2b green and blue lines). Only at 5× MIC of DCS was the response of LCS emulated (Figure 2b, red line). These data suggest that Alr inhibition leads to Ala depletion (as seen with LCS), while Ddl inhibition (by DCS at lower concentrations) causes Ala accumulation. At higher DCS concentrations, Alr is also inhibited, resulting in Ala depletion; hence, Ddl inhibition is occurring at lower DCS concentrations than is Alr inhibition. These lower concentrations of DCS are in the range of plasma levels obtained in humans treated with DCS[19] and therefore are significant to the effect observed during treatment.

To investigate the relative roles of Alr and Ddl in the mechanism of DCS action in more detail, we analyzed

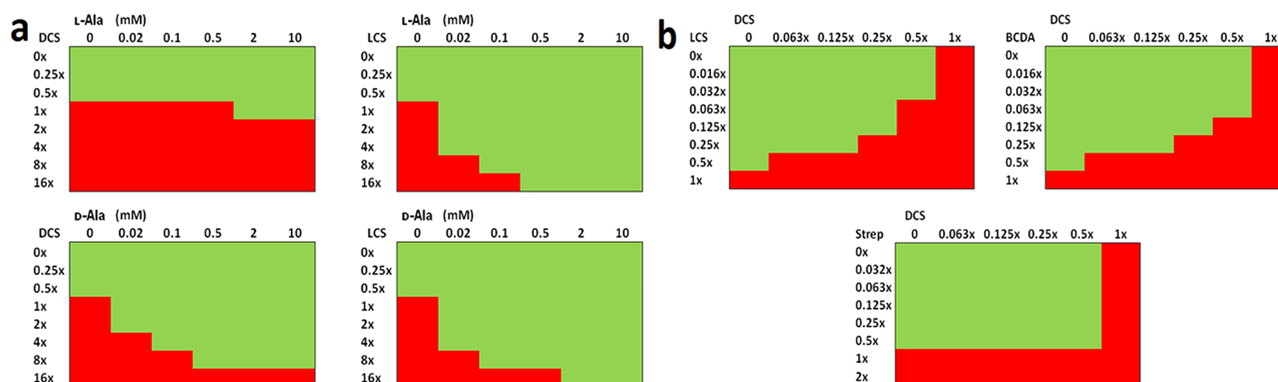



**Figure 1.** Growth scoring of *M. tuberculosis* H37Rv grown on 7H10 media containing varying concentrations of (a) DCS (left) or LCS (right) and L-Ala or D-Ala (top and bottom, respectively), or (b) DCS and other specified drugs. (n)× denotes multiplication of MIC of specified drug. Green indicates visible colonies, red indicates no visible growth. Growth was scored 7 days after initial inoculation. Results are representative of two independent assays.

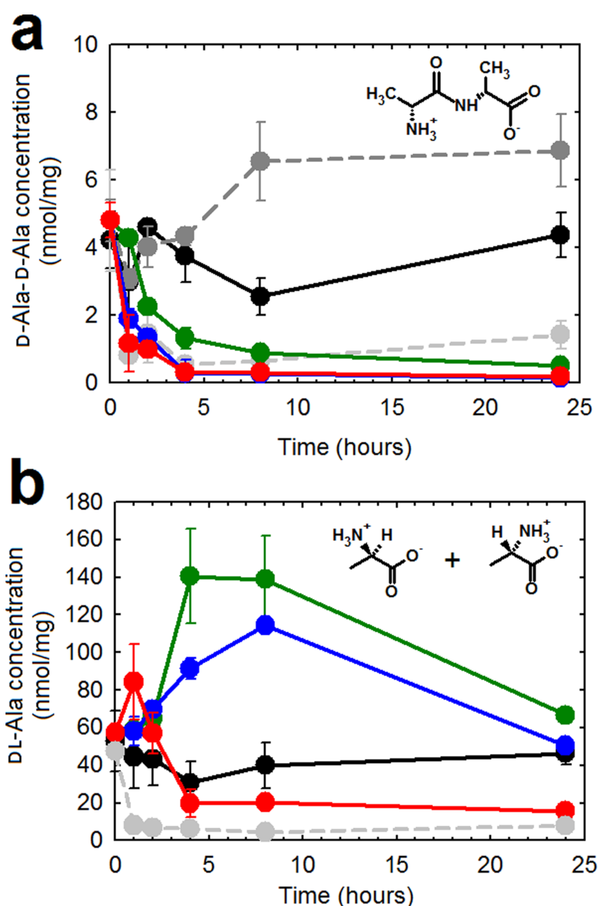



**Figure 2.** Changes in intracellular pool sizes of (a) D-Ala:D-Ala and (b) Ala (D- and L-) over 24 h following transfer of H37Rv-laden filters to 7H10 media supplemented with 0× (black), 0.25× (green), 1× (blue), or 5× (red) MIC of DCS, or 1× MIC of LCS (dashed light gray) or 5× MIC of streptomycin (dashed dark gray). *Y*-axis values are relative concentrations of target compounds normalized to the residual protein content of the respective sample (as a surrogate of cellular biomass). Data are the average ±1 standard deviation of duplicate samples.

metabolic flux through the D-Alanine pathway following DCS inhibition by employing $^{2}$H-isotopically labeled alanine. As Alr-catalyzed racemization proceeds through a two-base mechanism involving proton exchange between the $\alpha$ carbon and solvent,[20] we envisaged that racemization could be followed by observing the disappearance of the $\alpha$-$^{2}$H isotopologue over time (see Figure 3a,b). Cells were therefore challenged with increasing concentrations of DCS for 16 h before transfer to fresh media containing both DCS and either 1 mM L-Ala-$^{13}$C-$^{2}$H or 1 mM D-Ala-$^{2}$H. In the absence of DCS, the addition of L-Ala-$^{13}$C-$^{2}$H was accompanied by a simultaneous and rapid increase in the pool sizes of Ala+2 and Ala+1, indicating robust racemase activity under uninhibited conditions (Figure 3a,b, black lines). Correspondingly, flux of newly racemized D-Ala toward Ddl and incorporation of D-Ala-$^{13}$C3 into the dipeptide could be tracked by measuring pool sizes of D-Ala:D-Ala+1 and D-Ala:D-Ala+2 (shown as total D-Ala:D-Ala pool; Figure 3a right panel). Importantly, the presence of DCS at 1× MIC corresponded to a complete absence of higher order D-Ala:D-Ala isotopes (data not shown), suggestive of total inhibition of de novo D-Ala:D-Ala synthesis under these conditions. In contrast, a peak was observed in Ala+1 levels (Figure 3a middle panel, blue line) under the same conditions, albeit around 10% of uninhibited levels, indicating that Alr retains some activity at 1× MIC of DCS. In fact, racemization is slight but still evident at 5× MIC of DCS (Figure 3a middle panel, red line). The inability of low levels of newly racemized D-Ala to overcome DCS inhibition of Ddl provides solid evidence for a key role of Ddl inhibition in the antibiotic action of DCS.

Supplementation of DCS-inhibited bacteria with D-Ala-$^{2}$H generated complementary data, with parallel curves for D-Ala-$^{2}$H uptake (Ala+1 ion peak) and racemization (Ala ion peak) at 0×, 1×, and 5× MIC of DCS (Figure 3b, left and middle panels). Strong D-Ala:D-Ala ligase activity was also evident at 0× and 1× MIC (Figure 3b right), consistent with the ability of D-Ala to rescue DCS sensitivity by competitive binding to Ddl.

DCS has previously been shown to inhibit uptake of D-Ala (and vice versa) in multiple bacterial species, including *M. tuberculosis*, an effect believed to be a consequence of the shared transport system for the two compounds.[21] Therefore, D-Ala antagonism of DCS-induced growth inhibition could be due to decreased DCS uptake in the presence of exogenous D-Ala. We show here that this is not the case, as total intracellular DCS levels in *M. tuberculosis* grown on media containing both DCS and multiple concentrations of D-Ala are not only identical, but also higher than in bacteria grown without exogenous D-Ala (Figure S1, Supporting Information). Therefore, under these experimental conditions, DCS acts by inhibiting the synthesis of D-Ala:D-Ala and not uptake or racemization of Ala.

Several clinically used antibiotics display postantibiotic effects, whereby inhibition of cell growth continues after removal of the drug from the media or environment.[22] This is particularly important in vivo where effective drug concentrations fluctuate as a function of dosage, time, and clearance. We used our metabolomics approach to investigate how cells recovered following an initial DCS challenge and subsequent transfer to media lacking DCS. DCS levels drop to baseline (<10% of normal) levels within 15−60 min of transfer (Figure S2, Supporting Information); however D-Ala:D-Ala levels remain depleted for several hours post-DCS removal, and the time to recovery is dose-dependent with DCS; even after 24 h, D-Ala:D-Ala pool sizes do not recover to uninhibited levels (Figure 4). These results indicate for the first time that DCS displays a postantibiotic effect against *M. tuberculosis*, which might be partially responsible for the clinical efficacy of this drug against this bacterial species. We are currently unable to ascribe a definitive mechanistic basis for the observed postantibiotic effect; however, the recently described time-dependent inhibition of MtDdl by DCS may be partially responsible.[23] Specifically, the slow recovery of the D-Ala:D-Ala pool might indicate that MtDdl remains inhibited by DCS for long periods of time, after free DCS concentrations are undetectable in cells.

In summary, we have provided direct evidence, via an ex vivo stable isotope metabolomics approach, for a preferential or primary engagement of DCS with Ddl and therefore suggest a Ddl-centric mechanism of antibiotic action of DCS against *M. tuberculosis*. Inhibition of Alr activity also occurs, albeit not as strongly as Ddl, and more than likely synergizes with Ddl inhibition to cause a further decrease in metabolic flux through the D-Ala pathway. We therefore propose Ddl as a validated and practicable target for future drug development initiatives against *M. tuberculosis*.

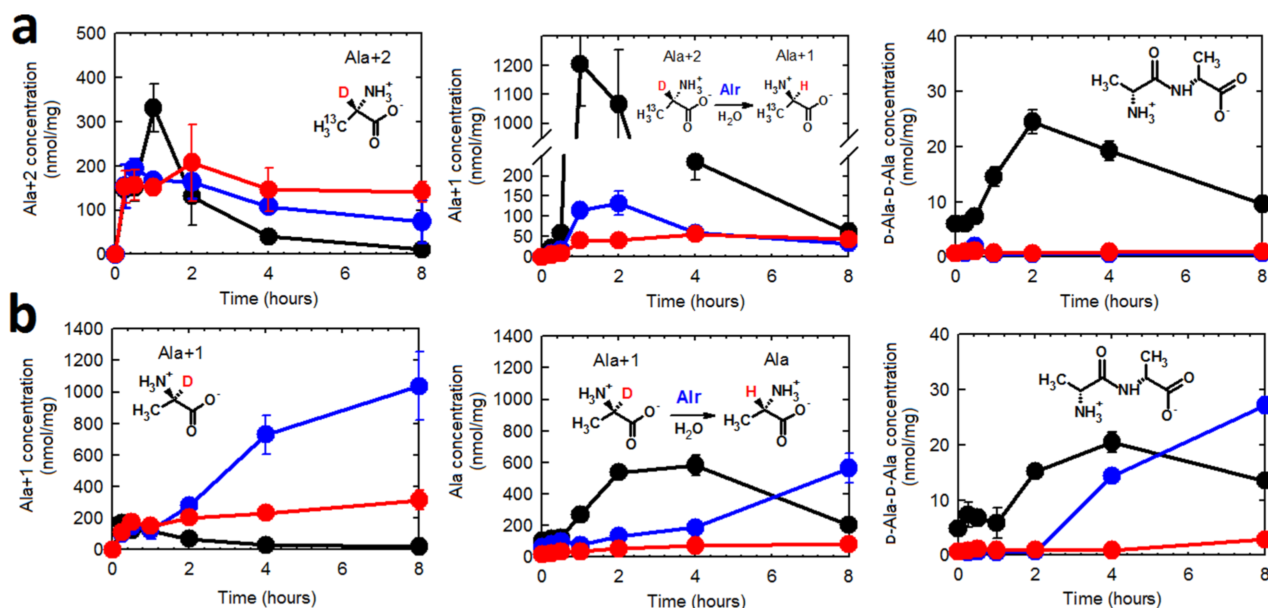



**Figure 3.** Changes in intracellular pool sizes of D-alanine pathway metabolites over 8 h, after transfer of H37Rv-laden filters to 7H10 media containing DCS and either (a) 1 mM L-Ala-$^{13}$C-$^{2}$H or (b) 1 mM D-Ala-$^{2}$H following a 16 h prior exposure to 0× (black), 1× (blue), or 5× (red) MIC of DCS. Metabolite levels followed are L-Ala-$^{13}$C-$^{2}$H (top left panel), Ala-$^{13}$C (top middle panel), D-Ala-$^{2}$H (bottom left panel), D,L-Ala (bottom middle panel), and D-Ala:D-Ala (top and bottom right panels). *Y*-axis values are relative concentrations of target compounds normalized to the residual protein content of the respective sample. Data are the average ±1 standard deviation of duplicate samples.

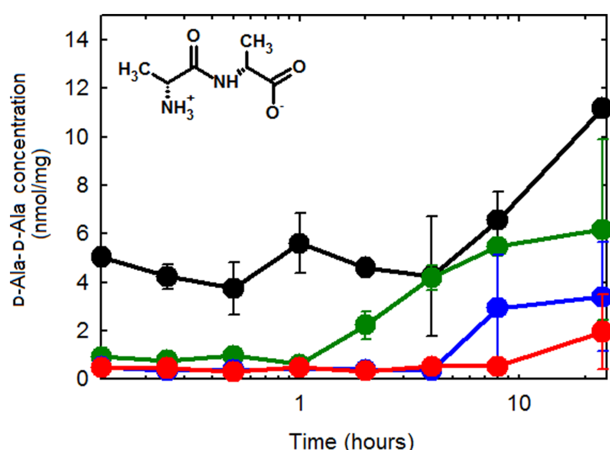



**Figure 4.** Changes in intracellular pool sizes of D-Ala:D-Ala over 24 h after transfer of H37Rv-laden filters to media lacking drug, following 16 h exposure to 0× (black), 0.25× (green), 1× (blue) ,or 5× (red) MIC of DCS. *Y*-axis values are relative concentrations of target compounds normalized to the residual protein content of the respective sample. Data are the average ±1 standard deviation of duplicate samples.

## ■ ASSOCIATED CONTENT

### Supporting Information

Material and Methods. Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

Alr, alanine racemase; BCDA, *β*-chloro-D-alanine; Ddl, D-alanine:D-alanine ligase; D-Ala:D-Ala, D-alanyl-D-alanine; D/LCS, D/L-cycloserine

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