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Inhibition of Mycobacterial Alanine Racemase Activity and Growth by Thiadiazolidinones

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Abstract

The genus *Mycobacterium* includes non-pathogenic species such as *M. smegmatis*, and pathogenic species such as *M. tuberculosis*, the causative agent of tuberculosis (TB). Treatment of TB requires a lengthy regimen of several antibiotics, whose effectiveness has been compromised by the emergence of resistant strains. New antibiotics that can shorten the treatment course and those that have not been compromised by bacterial resistance are needed. In this study, we report that thiadiazolidinones, a relatively little-studied heterocyclic class, inhibit the activity of mycobacterial alanine racemase, an essential enzyme that converts L-alanine to D-alanine for peptidoglycan synthesis. Twelve members of the thiadiazolidinone family were evaluated for inhibition of *M. tuberculosis* and *M. smegmatis* alanine racemase activity and bacterial growth. Thiadiazolidinones inhibited *M. tuberculosis* and *M. smegmatis* alanine racemases to different extents with 50% inhibitory concentrations (IC₅₀) ranging from <0.03 to 28 μM and 23 to >150 μM, respectively. The compounds also inhibited the growth of these bacteria, including multidrug resistant strains of *M. tuberculosis*. The minimal inhibitory concentrations (MIC) for drug-susceptible *M. tuberculosis* and *M. smegmatis* ranged from 6.25 μg/ml to 100 μg/ml, and from 1.56 to 6.25 μg/ml for drug-resistant *M. tuberculosis*. The *in vitro* activities of thiadiazolidinones suggest that this family of compounds might represent starting points for medicinal chemistry efforts aimed at developing novel antimycobacterial agents.

Keywords

D-alanine; alanine racemase; thiadiazolidinones; *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*

1. Introduction

The genus *Mycobacterium* includes more than 70 saprophytic and pathogenic species of Gram-positive aerobic bacteria [1]. With characteristics such as acid-fastness, a G-C-rich

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genome, and mycolic acid cell wall, this genus is divided into two groups on the basis of growth rate on solid medium. Slowly growing mycobacteria (SGM) such as *M. tuberculosis* form visible colonies after more than seven days, while rapidly growing mycobacteria (RGM) such as *M. smegmatis* form colonies in fewer than seven days [2].

Both SGM and RGM include medically important pathogens. The SGM with a significant medical burden is *M. tuberculosis*, the causative agent of tuberculosis (TB), which afflicts nearly nine million people and kills more than one million people yearly [3]. Mycobacterial infections are difficult to treat due to the lipid-rich cell envelope that often confers intrinsic drug resistance in these bacteria [4]. Standard therapy consists of a lengthy regimen of antibiotic cocktails, which though often curative, suffers from poor patient compliance and diminished effectiveness due to the emergence of drug resistance [5]. There are currently over 500,000 cases of multidrug resistant TB (MDR-TB) for which standard therapy is not effective, requiring second-line drugs that are often toxic and of questionable efficacy [6]. Progress in antimycobacterial drug development is slow. The ATPase inhibitor, bedaquiline, which was recently approved by the FDA for treatment of drug-resistant TB, is the first novel TB drug in nearly half a century [7–9]. A number of other compounds are in clinical development [10]. To ensure a continuous pipeline of new drugs that are effective against drug resistant strains and can shorten therapy, additional efforts are necessary to identify new bacterial drug targets or reevaluate old targets.

One mycobacterial target of significant historical interest is alanine racemase [11–13], a pyridoxal phosphate (PLP)-containing homodimeric enzyme that catalyzes the conversion of L-alanine to D-alanine for the synthesis of the peptidoglycan layer in bacterial cell walls [14]. In mycobacteria, the approximately 41 kDa monomer of alanine racemase is encoded by a unique *alr* gene whose inactivation leads to a D-alanine auxotrophic phenotype that is impaired in intracellular growth and viability [15–17]. The essentiality of the *alr* gene coupled with a lack of a known homolog in humans make alanine racemase an attractive target for antimycobacterial agents. Cycloserine, a natural antibiotic produced by *Streptomyces* species, is known to target this enzyme [12, 18]. Though used successfully to treat drug resistant TB in the past, the utility of cycloserine in TB therapy is limited due to drug-induced neurotoxicity [19–21]. Cycloserine is a cyclic analog of alanine and its mechanism of enzyme inactivation occurs through covalent adduct formation with the PLP cofactor [22]. Potential toxicity due to off-target effects of cycloserine and other substrate analogs have prompted efforts to identify alanine racemase inhibitors that are structurally unrelated to alanine.

We recently reported results from a high throughput screening effort that identified several novel classes of alanine racemase inhibitors with antimicrobial activities [23, 24]. Here we report the activity of thiadiazolidinones, a novel class of alanine racemase inhibitor, against the enzymes of *M. tuberculosis* and *M. smegmatis* as well as their effect on the growth of these bacteria, and discuss the potential of this class of compounds as antimycobacterial agents.

2. Materials and Methods

2.1. Bacterial strains and growth media

E. coli strain DH5 α (New England Biolabs, Ipswich, MA) used for plasmid propagation and *E. coli* strain Rosetta-2 (plys) DE3 (EMD Millipore, Rockland, MA) routinely used for protein expression, were grown in LB medium containing 100 μ g/ml ampicillin. The alanine racemase deficient *E. coli* strain MB2946 (Δ (*araA-leu*) 7697, [*araD139*]_{B/r}, Δ (*codB-lacI*)3, *galK16*, *galE15*(*GalS*), λ^- , *e14*⁻, *dadX100::FRT*, *relA1*, *rpsL150*(*strR*), *spoT1*, *alr-100::FRT*, *mcrB1* [25]) was obtained from Yale University *E. coli* Genetic Stock Center

and was maintained in LB medium containing 10 µg/ml streptomycin and 50 mM D-alanine. *M. tuberculosis* Erdman ATCC 35801, *M. tuberculosis* H37Rv, and *M. smegmatis* ATCC 700084 were purchased from the American Type Culture Collection (Manassas, VA). The MDR strains are clinical isolates from Dr. Cynamon's collection. *M. tuberculosis* isolates were grown in modified 7H10 broth (pH 6.6; 7H10 agar formulation with agar and malachite green omitted) with 10% OADC (oleic acid, albumin, dextrose, catalase) enrichment (BBL Microbiology Systems, Cockeysville, MD) and 0.05% Tween 80 for 5–10 days on a rotary shaker at 37°C. *M. smegmatis* was grown in Mueller Hinton Broth (BBL Microbiology Systems) for 2 days on a rotary shaker at 37°C.

2.2. Chemicals

Thiadiazolidinones originated from the Maybridge Screening library and were purchased from Fisher Scientific (Pittsburgh, PA). Ampicillin, Streptomycin, D- and L-alanine, PLP, and β-NAD sodium salt, and cycloserine were purchased from Sigma Aldrich (St. Louis, MO).

2.3. Cloning, expression and purification of recombinant *M. smegmatis* and *M. tuberculosis* alanine racemases

The *alr* genes of *M. smegmatis* strain MC² 155 (NCBI Reference Sequence: **YP_885954.1**) and *M. tuberculosis* H37Rv (*alr*_{TB}-short as reported in [25] and NCBI Reference Sequence: **NP_217940**) were used as templates for constructing synthetic DNA expression constructs, encoding the 389 and 384-amino acid proteins, respectively, fused to N-terminal-hexahistidine tags (GenScript USA Inc., Piscataway, NJ). The constructs, which had codons optimal for expression in *E. coli*, were cloned into the *Xba*I/*Bgl*II sites of pET32a vector (Novagen, Madison, WI). Plasmids were transformed into *E. coli* strain Rosetta-2 (plys) DE3, by heat shock method, and successful transformation was confirmed by colony PCR using T7 promoter and T7 terminator primers (EMD Millipore, Rockland, MA). Initial small-scale expression screens were performed on selected positive clones by culturing a colony in 10 ml LB plus 100 µg/ml ampicillin to OD₆₀₀ of 0.8. Protein induction was initiated by the addition of IPTG to a final concentration of 0.4 mM for 16 hours at 28 °C. For large scale expression, 1.2 L cultures were grown in 2.8 L Fernbach flask under this condition. Cell pellets were harvested by centrifugation, suspended in lysis buffer (85ml PBS + 5mL 100% TritonX-100, 10 ml 100% glycerin, and Roche complete EDTA-free protease inhibitor cocktail), and lysed under high pressure in an Emulsiflex homogenizer (Avestin Canada). The protein in the clarified supernatant was purified by affinity chromatography on a Ni-FF crude column (GE Healthcare, Piscataway, NJ) using phosphate buffer saline pH 7.4 eluted with 250 mM imidazole. Protein was dialyzed in the storage buffer (50 mM Tris pH 8.0, 1 mM DTT, 10 µM PLP) and stored at –80 °C.

2.4. Alanine racemase assay and IC₅₀ determination

Kinetic parameters of alanine racemase activity i.e. the rate of conversion of L-alanine to D-alanine, were determined by measuring the amounts of the two enantiomers by HPLC. Reaction mixtures consisting of 0.5 µg *M. tuberculosis* or 0.007 µg *M. smegmatis* alanine racemases in 10 mM borate buffer pH 8.5 and 5 mM L-alanine substrate were incubated for 5 minutes at room temperature. Enzyme reaction was stopped by adding four volumes of methanol to the reaction mixture. L and D-alanine in the methanol extracts were separated by means of isocratic HPLC (Agilent 1100 Chemstation) with a Nucleosil-Chiral-1 column (Machery & Nagel, Germany) that separates the enantiomers on the basis of their differential diastereomeric stability. A 0.5 mM copper sulfate solution was used as the mobile phase at a flow rate of 0.3 ml/min and a column temperature of 60 °C. Percentage of the D-alanine product of total L- and D-alanine was calculated by peak area from the HPLC

profile at A_{240nm}. For IC₅₀ determination, various concentrations of inhibitors (prepared at 5 mg/ml in ethanol/DMSO 50:50 volume) were pre-incubated with the enzymes for 30 minutes prior to HPLC analysis.

2.5. Complementation analysis

For complementation analysis, plasmids in which the *alr* genes were constitutively expressed from the *lac* promoter were constructed by inserting the *M. smegmatis* and *M. tuberculosis alr* genes into the *Xba*I-*Sac*I sites of the pUC57 vector. The plasmids were electroporated into the alanine racemase-deficient *E. coli* strain MB2946 [25], an *alr/dadX* double mutant whose growth is dependent on D-alanine supplementation in the medium. Transformants were selected on LB medium without added D-alanine supplementation to assess growth rescue.

2.6. Electrospray ionization mass spectrometry (ESMS) and comparative MALDI-MS

ESMS was performed to detect direct interactions of the inhibitor with the enzyme. A mixture of 15 µl of sample (4 µM enzyme with 1 mM inhibitor) was diluted with 15 µl 2% acetonitrile/ 0.1% formic acid. Samples were then de-salted using C4 ZipTips, eluted into 15 µl 60% acetonitrile/ 0.1% formic acid, and directly injected into a Q-TOF-micro MS instrument (Waters Corp., Milford, MA). To identify potential modified residues, samples were prepared as described for the ESMS. Five microliter of the desalted material was denatured in 8 M urea/0.4 M NH₄HCO₃ (Sigma Aldrich), alkylated with 14 mM iodoacetic anhydride (Sigma Aldrich), digested overnight with trypsin, 1:25 (Sigma Aldrich), and kept frozen until further use. The samples were analyzed by MALDI-MS on a MDS SCIEX 4800 MALDI TOF/TOF Analyzer (Applied Biosystems).

2.7. In vitro antimycobacterial minimal inhibitory concentration (MIC) studies

MIC analysis was performed using the Microtiter Broth Dilution Assay as previously described [23]. Briefly, 2.5×10^5 CFU/ml of *M. tuberculosis* and *M. smegmatis* were plated in 50 µl of modified 7H10 broth and Mueller Hinton broth, respectively in 96 well round-bottom microtiter plates (Corning Inc., Corning, NY). Serial dilutions of the study drugs are prepared in broth, and 50 µl of the broth was added to the wells containing the bacteria to yield a final volume of 100 µl. The plates were covered with SealPlate adhesive sealing film (Excel Scientific, Wrightwood, CA) and incubated at 37°C in ambient air for 18–21 days for *M. tuberculosis* and 2 days for *M. smegmatis* and growth or lack thereof was assessed by visual inspection. The MIC was defined as the lowest concentration of antimicrobial agent yielding no visible turbidity.

2.8. Compound-induced cytotoxicity assays

The cytotoxicity of thiadiazolidinones was assessed by incubating HeLa cells (CCL-2, ATCC, Manassas, VA) with an eight-point dilution series of the inhibitors (concentrations ranging from 100 µM to 0.78 µM) in culture medium in 96-well plates for 48 hours. Following this, cell viability was assessed by the lactate dehydrogenase (LDH) release assay (Promega Corp., Madison, WI) according to manufacturer's recommendations.

3. Results

3.1. *M. smegmatis* and *M. tuberculosis* alanine racemases are highly homologous enzymes with markedly different catalytic activities

The genomes of the RGM *M. smegmatis* [26] and SGM *M. tuberculosis* [27] each contain one *alr* gene. The gene products are highly homologous showing 65% identity in their primary sequence (Figure 1A). Structurally, the two enzymes seem to share an almost

identical fold, with two monomers in head-to-tail orientation forming a dimer with two active sites that contain the PLP-binding lysine (Lys₄₆ and Lys₄₂ in *M. smegmatis* and *M. tuberculosis*, respectively) ([28] and Figure 1B).

To facilitate the expression of the GC-rich (65–71%) mycobacterial *alr* genes in a heterologous host, synthetic genes with codons optimized for expression in *E. coli*, were chemically synthesized. The functionality of the synthetic constructs was first confirmed in an *E. coli* D-alanine auxotroph, where the synthetic *alr* genes complemented the alanine racemase mutation and restored growth of the mutant in D-alanine deficient medium (Figure 2A). Upon confirmation of their biological activity, high-levels of recombinant alanine racemases were produced in *E. coli* and purified to homogeneity (Figure 2B). To measure the ability of the recombinant enzymes to racemize L-alanine to D-alanine, an HPLC-based method utilizing a chiral column to separate the enantiomers based on their differential diastereomeric stability, was developed. Both recombinant alanine racemase enzymes were shown to be active by this method. Figure 2C shows the kinetic plots for the two enzymes. *M. smegmatis* alanine racemase showed a higher catalytic activity with a K_m for L-alanine of 8.5 mM and V_{max} of 0.3 mM/min and K_{cat} of 4300. The *M. tuberculosis* enzyme displayed slower kinetics with K_m for L-alanine of 4 mM, V_{max} of 0.04 mM/min and K_{cat} of 200, consistent with previous reports [25, 28].

3.2. *M. smegmatis* and *M. tuberculosis* alanine racemases are differentially inhibited by thiadiazolidinones

Considering the vastly different catalytic activities of the two recombinant enzymes, we examined the ability of thiadiazolidinones to inhibit the activities of the two enzymes. We chose twelve members of the thiadiazolidinone family, each with an intact thiadiazolidinone core but with various substitutions at the N-linked groups (Figure 3). The IC₅₀ values for each of the thiadiazolidinone and cycloserine control are shown in Table 1. In all instances, the thiadiazolidinones inhibited *M. tuberculosis* alanine racemase to a greater extent than the *M. smegmatis* enzyme. The IC₅₀ values for the *M. tuberculosis* enzyme ranged from <0.03 to 28 μ M with all but one thiadiazolidinone (401-4) showing good inhibitory activity (IC₅₀ < 10 μ M). The IC₅₀ values for the *M. smegmatis* enzyme ranged from 0.43 to >150 μ M, but unlike the *M. tuberculosis* enzyme, only the thiadiazolidinone 407-7, had good inhibitory activity against the *M. smegmatis* enzyme. A similar trend was observed with cycloserine.

We proceeded to examine whether the marked difference in the inhibition potency of thiadiazolidinones against the two alanine racemases was due to differential binding. We selected 401-11 (IC₅₀ *M. smegmatis* alanine racemase >100 μ M and *M. tuberculosis* alanine racemase of 0.13 μ M, and molecular weight of 316) and examined its ability to bind the enzymes by mass spectrometry. Figure 4 shows the ESMS spectra of the enzymes with and without the inhibitor. The inhibitor induces an upward shift in both *M. tuberculosis* and *M. smegmatis* enzyme monomer peaks corresponding approximately to the molecular weight of a single inhibitor molecule, indicating binding. The ESMS data thus suggest that differential inhibition of alanine racemase activity by thiadiazolidinones is likely not due to lack of inhibitor binding.

Next, we attempted to identify the site on the enzymes where the inhibitor bound. Inhibitor-reacted enzyme complexes were subjected to proteolytic digestion by trypsin and peptide mapping by MALDI-TOF mass spectrometry. Peptide mapping produced more than 90% sequence coverage lacking only thirteen amino acids at the C-terminus. The MS spectra for samples with and without the inhibitor were overlaid and carefully examined for peaks unique to the inhibitor-containing samples. The spectra were virtually superimposable with no potential adduct peaks identified in the inhibitor-treated samples (data not shown), revealing no stable covalent modification by the inhibitor. Particular care was taken to

investigate all cysteine-containing peptides since thiadiazolidinones are thought to potentially covalently bind to cysteine residues [29]. No compound-induced modification was observed at any of the three cysteine residues recovered in the peptide mapping. The absence of a modification in the inhibitor-treated sample suggests that the inhibitor likely binds the enzyme non-covalently and that this interaction is likely disrupted during sample preparation.

3.3. Inhibition of *M. smegmatis* and *M. tuberculosis* growth by thiadiazolidinones

Since thiadiazolidinones inhibited *M. tuberculosis* and *M. smegmatis* alanine racemases to different extents, we proceeded to examine whether the differences extended to inhibition of growth of these bacteria as well. We determined the MICs of the twelve thiadiazolidinones along with the cycloserine control antibiotic against *M. tuberculosis* and *M. smegmatis*. As summarized in Table 1, all twelve thiadiazolidinones were active against *M. tuberculosis* with MICs ranging from 6.25 to 25 µg/ml. The MICs for *M. smegmatis* ranged from 25 to 100 µg/ml with the exception of 401-2, where the MIC was 0.39 µg/ml. In comparison, the MICs for cycloserine were 6.5 µg/ml and 25 µg/ml for *M. tuberculosis* and *M. smegmatis*, respectively. The thiadiazolidinone 401-11 was evaluated further for activity against five clinical isolates of MDR *M. tuberculosis*, and was shown to inhibit the growth of all five strains with MICs ranging from 1.56 to 6.25 µg/ml (Table 2), which were significantly lower than that of cycloserine. Overall, the data suggested that the vast difference observed in inhibition of the two alanine racemase activities by the thiadiazolidinones did not extend to growth inhibition. Therefore, we wanted to determine if thiadiazolidinones inhibited growth by directly blocking the cellular function of alanine racemase. The cellular effect of a particular inhibitor can often be mitigated or lessened by exogenously supplying the product of the target. Therefore, we measured MICs of the thiadiazolidinones in the presence of 5 mM D-alanine in the growth medium. While a 3-fold increase in MIC was observed for cycloserine no significant increase was observed for the thiadiazolidinones (data not shown), indicating that these inhibitors likely have other cellular targets.

3.4. Mammalian cell toxicity of thiadiazolidinones

We further examined the effect of thiadiazolidinones on the human HeLa cell line to determine if the observed antimicrobial activity could be due to non-specific compound-induced cellular toxicity. Using an assay that measures the release of endogenous lactate dehydrogenase enzyme, we gauged the extent to which thiadiazolidinones breached membrane integrity or caused cell lysis. After 48 hours of exposure, eight of the inhibitors (401-1, 3, 4, 5, 7, 9, 10–12) did not cause significant cytotoxicity (Figure 5) even at 100 µg/ml, while four of the inhibitors (401-2, 3, 6, and 8) appear to be moderately toxic with TC₅₀ values (concentration of compound causing 50% cell lysis) greater than 100 µg/ml. Since the TC₅₀ values for the majority of the inhibitors are higher than the MIC values, the antimicrobial activity might not be due to general toxicity.

3.5 Preliminary structure-activity relationship (SAR) of thiadiazolidinones

To confirm whether the inhibition of alanine racemase and growth activities of thiadiazolidinones are related, we performed a preliminary SAR for this series. Comparison of enzyme IC₅₀ values and MICs revealed that greater potency of alanine racemase inhibition does not necessarily correlate with greater cellular inhibitory potency. For instance, 401-7, which is the most potent enzyme inhibitor (IC₅₀ <0.5 µM), has modest antimicrobial activity (MIC 25–50 µg/ml), while 401-2, which is a relatively poor enzyme inhibitor (IC₅₀ of 8 µM and >100 µM against *M. tuberculosis* and *M. smegmatis* alanine racemases, respectively), has potent antimicrobial activity (MICs 6.25 µg/ml *M. tuberculosis* and 0.39 µg/ml *M. smegmatis*). These results suggest that growth inhibition mediated by

thiadiazolidinones is likely not due to direct inhibition of cellular alanine racemase alone, and that these inhibitors likely function by other mechanisms.

4. Discussion

As an enzyme essential for the construction of cell wall in mycobacteria, alanine racemase is an attractive and validated target for antimycobacterial drug development. Here we report the activity of the thiadiazolidinone family against the homologous enzymes of *M. smegmatis* and *M. tuberculosis*, and additionally examine their antimycobacterial activities against these bacteria. Our studies reveal a surprising finding that thiadiazolidinones differentially inhibit the activities of the two mycobacterial alanine racemases. While the thiadiazolidinone family shows promising antimycobacterial activities, growth inhibition does not appear to be a direct consequence of inhibition of cellular alanine racemase.

Eleven of the twelve members of the thiadiazolidinone family showed potent activity ($IC_{50} < 10 \mu M$) against the *M. tuberculosis* enzyme, but their activity against the *M. smegmatis* enzyme was greatly diminished. Since ESMS analyses indicated that thiadiazolidinones bind both enzymes, the physical basis of the differential inhibition is not evident, but likely tied to the differences in the catalytic activities of these enzymes. It is intriguing that despite a high degree of sequence conservation, the *M. tuberculosis* enzyme is significantly less active than the *M. smegmatis* enzyme. Differences in the catalytic activities of alanine racemases have been reported in bacteria that harbor two isoforms of this enzyme. For instance, in *E. coli*, *P. aeruginosa*, and *S. typhimurium* the *alr* and *dadX* genes code for two alanine racemase isoforms. The *alr* gene is constitutively expressed at low levels and encodes the enzyme for cell wall synthesis. The *dadX* gene is induced when L-alanine is abundant in the medium, and the D-alanine generated is oxidized to pyruvate for use as carbon and energy source [30–33]. Consistent with the high rate of turnover necessary to metabolize excess L-alanine, the DadX enzyme often displays higher catalytic activity than the Alr enzyme despite a high degree of sequence homology [33]. Variations in catalytic activities of alanine racemases have been attributed to the differences in the monomer-dimer equilibrium. The more active isoforms are thought to exist predominantly as catalytically active dimers due to the increased propensity of the monomers to associate, while the less active isoforms, with their lower monomer association constants are largely monomeric [34]. Accordingly, the differences in the monomer-dimer equilibrium between the *M. smegmatis* and *M. tuberculosis* enzymes could explain the differences in their susceptibility to thiadiazolidinones. In the case of *M. smegmatis* alanine racemase, the binding of the inhibitor to the predominantly dimeric enzyme might not greatly impact its activity, whereas in the *M. tuberculosis* enzyme, the inhibitor binding to the predominantly monomeric enzyme might prevent its association to the dimer form.

The molecular mechanism by which thiadiazolidinones inactivate mycobacterial alanine racemases still needs to be established. Nevertheless, the structural features of thiadiazolidinones, including the lack of primary amines and the size, suggest that this series are unlikely to interact with the enzyme-bound PLP cofactor or to occupy the substrate-binding site due to spatial constraints within the active site, which can only accommodate molecules within the size range of alanine (89 MW). Therefore, thiadiazolidinones likely block alanine racemase activity by a mechanism other than active-site binding. It has been suggested that in general thiadiazolidinones covalently inactivate enzymes by nucleophilic attack of cysteine residues [29]. However, the mass spectrometry results, which revealed no covalent modification of the cysteine residues, suggested a non-covalent interaction between the inhibitor and the enzyme. Structural studies with enzyme-inhibitor co-crystals are necessary to elucidate the nature of thiadiazolidinone and alanine racemase interactions.

Several thiadiazolidinones demonstrated good antimicrobial activity against *M. tuberculosis*, including MDR strains, but their activity against *M. smegmatis* growth was relatively modest. The differential susceptibility could be partly due the vastly different growth rates of these bacteria; the generation times for *M. tuberculosis* is 24 hours while that of *M. smegmatis* is 3–4 hours [35]. Despite these differences, the antimicrobial activity of thiadiazolidinones is evident. We have previously reported that this family of compounds selectively inhibits Gram-positive bacteria, yeast and fungi, but not Gram-negative bacteria [24]. Others have reported general antibacterial and antiviral properties of thiadiazolidinones though the mechanism of action still remains unknown [36, 37]

Thiadiazolidinone-mediated growth inhibition, however, does not appear to arise solely from inhibition of cellular alanine racemase as the inhibitory effect could not be mitigated by supplying D-alanine exogenously. These results suggest the existence of additional cellular targets, a notion supported by reports of activities of this family of compounds on other non PLP-dependent enzymes. For instance, thiadiazolidinones have been reported to inhibit the *de novo* pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase [29], the ATPase CagA component of the contact-dependent secretion system in *Helicobacter pylori* [38], as well as the RGS4 member of the eukaryotic regulators of G protein signaling [39]. Due to these potential off-target effects, additional medicinal chemistry efforts are necessary to increase the selectivity of thiadiazolidinones in order to further their development as antimicrobials.

The development of antimicrobials targeting alanine racemase has been hampered by the lack of safe and highly specific inhibitors. Non-substrate inhibitors of this enzyme such as the thiadiazolidinone family reported in this study, have only recently come to light [23, 24]. Thiadiazolidinones, which are structurally distinct from the active-site binder, cycloserine, could potentially reveal novel strategies of inhibiting alanine racemase, and pave the way for the rational design of more selective inhibitors.

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A

<i>M. smegmatis</i> /1-389	MQTTEPMTPPAFLASQTVIDLGAIDHNVRVLRRELAGSADVMAVVKADAYGHGALPVARTALAGAAALGVATIPALALREGGITAPVLAWLHPPGT	98
<i>M. tuberculosis</i> /1-384	--MTPIIS-QTRGLLEAMVDLGAIENHVRVLRREHAGHAOLMAVVKADGYGHGATRVAAQTALGAGAAELGVATVDEALALRADGITAPVLAWLHPPGI	94
<i>M. smegmatis</i> /1-389	DFAPAIADVEVAVSSRRQLEQVTAAAEVGRATVTVKVDTGLSRNGVGAADYFEVLDVLRRAQADGAIRVRLMSHLVHGDDPENPFNGLGGORLA	196
<i>M. tuberculosis</i> /1-384	DFGPALLADVQAVSSLRQLEDELLHAVRRRTGRATVTVKVDTGLENRNGVGAQFFAMLTALRQAAEDAVRLRGLMSHMYADKPDSSINDVQAQRF	192
<i>M. smegmatis</i> /1-389	DMRVYAREHGVDEVAHLNCSPAAMTRPDLAEMVRPGISLYGLSPIFERGDMLRPAMTLKCPVALVRSVHAGDGVSYGHRVVAARDTTGLLPFGY	294
<i>M. tuberculosis</i> /1-384	AFLAQAREQGVRF EVAHLSNSATMARPDLTDLVRPGIAVYGLSPVFAALGDMGLVPAMTVKCAVALVKSIRAGEGVSYGHTWIAPRDTNLALLPIGY	290
<i>M. smegmatis</i> /1-389	ADGVYRALSGRIDVLIKRRRRRAVGRICMDQFVVDLGPDAADVAVGDDAILFGPGANGEPATAQDWAELLDTIHYEVVTSFRGRVTRTYLPAGQDD	389
<i>M. tuberculosis</i> /1-384	ADGVFRSLGGRLEVLINRRCPGVGRICMDQFMVDLGPGLDVAEGDEAILFGPGIRGEPTAQDWAOLVGTIHYEVVTSFRGRITRTYREANR-	384

B

Figure 1. Sequence comparison of *M. smegmatis* and *M. tuberculosis* alanine racemases
 (A) ClustalW alignment of *M. smegmatis* and *M. tuberculosis* alanine racemase sequences. Identical residues are shaded while the PLP-binding lysine is shown with an asterisk. (B) Ribbon diagram of the *M. tuberculosis* (Mtb) alanine racemase dimer (pdb code 1XFC) with helices in red, strands in yellow and loops in green. The gray sticks represent the side chain of Lys₄₂ and the PLP cofactor and arrows indicate the two active sites. The homology model of *M. smegmatis* (MSM) alanine racemase (right) was generated based on the structure of Mtb alanine racemase using SWISS_MODEL WORKSPACE. The same color scheme is used and the gray sticks represent the side chain of Lys₄₅ and the PLP cofactor.

Figure 2. Activity comparison of recombinant *M. smegmatis* and *M. tuberculosis* alanine racemases

(A) Growth rescue of a D-alanine auxotroph by plasmids expressing recombinant *M. tuberculosis* and *M. smegmatis* alanine racemases. Growth or lack thereof of (a) *E. coli* MB2159, (b) transformed with *M. tuberculosis* *alr*, (c) *M. smegmatis* *alr*, and (d) pUC57 vector on LB medium supplemented with 50 mM D-alanine (+ D-alanine) and LB medium without supplementation (– D-alanine). (B) Purification of recombinant *M. tuberculosis* (Mtb) and *smegmatis* (MS) alanine racemases. Coomassie Blue-stained SDS-PAGE gel showing proteins from nickel affinity column. Arrows indicate the ~41,000 MW bands corresponding to monomeric alanine racemases. M is molecular weight markers. (C) Enzyme activity plot of purified alanine racemases.

401-1:	R ₁ = cyclopropylmethyl;	R ₂ = 3,5-dimethyl-isoxazole-4-yl
401-2:	R ₁ = 2,3-dihydro-1 <i>H</i> -inden-2-yl;	R ₂ = 3-chloro-4-fluorophenyl
401-3:	R ₁ = 2,3-dihydro-1 <i>H</i> -inden-2-yl;	R ₂ = 2-chloro-4-nitrophenyl
401-4:	R ₁ = 2,3-dihydro-1 <i>H</i> -inden-2-yl;	R ₂ = ethyl-3-benzoyl
401-5:	R ₁ = cyclopropylmethyl;	R ₂ = 2-chloro-6-methylphenyl
401-6:	R ₁ = cyclopropylmethyl;	R ₂ = 2-trifluoromethylphenyl
401-7:	R ₁ = cyclopropylmethyl;	R ₂ = 2-chloro-4-nitrophenyl
401-8:	R ₁ = methyl;	R ₂ = 1-naphthyl
401-9:	R ₁ = methyl;	R ₂ = phenyl
401-10:	R ₁ = 4-isopropylphenyl;	R ₂ = 4-(trifluoromethylthio)phenyl
401-11:	R ₁ = 4-fluorobenzyl;	R ₂ = <i>p</i> -tolyl
401-12:	R ₁ = 4-fluorophenyl;	R ₂ = 3,5-dimethyl-isoxazole-4-yl

Figure 3. Structure of the thiadiazolidinone core and 12 analogs with various N-linked substitutions

Figure 4. Analysis of alanine racemase and thiadiazolidinone interaction
(A) ESMS spectra of *M. tuberculosis* (Mtb) *M. smegmatis* (MSM) alanine racemases alone (top) and in complex with 401-11 (bottom). Arrows and asteriks indicate the shift in the alanine racemase monomer peaks by approximately 316 Da.

Figure 5. Analysis of thiadiazolidinone-induced cytotoxicity in HeLa cells

Cells were exposed to various concentrations of compounds for 48 hours after which the extent of cell lysis was measured by the LDH-release assay. The plot shows percent LDH released of each compound with respect to a DMSO-treated control.

Inhibition of mycobacterial Alr activity and growth by thiadiazolidinones and cycloserine (CS)

Table 1

Cpd.	Structure	Mtb Alr IC ₅₀ (μ M)	MSM Alr IC ₅₀ (μ M)	Mtb MIC (μ g/mL)	MSM MIC (μ g/mL)
401-1		1.46	>100	25	50
401-2		8.46	>100	6.25	0.39
401-3		0.05	>100	12.5	50
401-4		28.76	>100	6.25	50
401-5		4.48	>100	12.5	100
401-6		0.82	16.6	25	50

Cpd.	Structure	Mtb A/R IC ₅₀ (μ M)	MSM A/R IC ₅₀ (μ M)	Mtb MIC (μ g/mL)	MSM MIC (μ g/mL)
401-7		<0.03	0.43	25	50
401-8		0.12	20.09	12.5	25
401-9		0.29	25.53	6.25	25
401-10		0.17	>100	12.5	25
401-11		0.13	>100	25	25
401-12		0.07	68.01	25	100
CS		0.7	>100	6.25	25

Table 2

In vitro antimycobacterial activities of thiadiazolidinone 401-11 and cycloserine (CS) against drug resistant strains of *M. tuberculosis* as revealed by the MIC required to inhibit growth.

<i>M. tuberculosis</i> Strain	Resistance	MIC (µg/ml)	
		401-11	CS
H37Rv	Fully susceptible	3.125	6.25
Beijing W MDR	INH ^R , Rif ^R , Str ^R , EMB ^S , Gat ^S	1.56	50
Beijing F29 MDR	INH ^R , Rif ^R , Str ^S , EMB ^S , Gat ^S	3.125	25
Clinical MDR	INH ^R , Rif ^R , Str ^R , EMB ^R , Gat ^S	3.125	25
Clinical MDR	INH ^R , Rif ^R , Str ^R , EMB ^R , Gat ^S	6.25	12.5
Clinical MDR	INH ^R , Rif ^R , Str ^R , EMB ^R , Gat ^S	3.125	50

INH^R- resistance to isoniazid as defined by MIC > 0.1 µg/ml

Rif^R- resistance to rifampin as defined by MIC >0.5 µg/ml

Str^R- resistance to streptomycin as defined by MIC >2 µg/ml

EMB^R- resistance to ethambutol as defined by MIC > 4 µg/ml

GAT^R- resistance to gatifloxacin as defined by MIC >0.5 µg/ml