



Revisiting the β -Lactams for Tuberculosis Therapy with a Compound-Compound Synthetic Lethality Approach

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ABSTRACT The suboptimal effectiveness of β -lactam antibiotics against *Mycobacterium tuberculosis* has hindered the utility of this compound class for tuberculosis treatment. However, the results of treatment with a second-line regimen containing meropenem plus a β -lactamase inhibitor were found to be encouraging in a case study of extensively drug-resistant tuberculosis (M. C. Payen, S. De Wit, C. Martin, R. Sergysels, et al., *Int J Tuberc Lung Dis* 16:558–560, 2012, <https://doi.org/10.5588/ijtld.11.0414>). We hypothesized that the innate resistance of *M. tuberculosis* to β -lactams is mediated in part by noncanonical accessory proteins that are not considered the classic targets of β -lactams and that small-molecule inhibitors of those accessory targets might sensitize *M. tuberculosis* to β -lactams. In this study, we screened an NIH small-molecule library for the ability to sensitize *M. tuberculosis* to meropenem. We identified six hit compounds, belonging to either the *N*-arylindole or benzothiophene chemotype. Verification studies confirmed the synthetic lethality phenotype for three of the *N*-arylindoles and one benzothiophene derivative. The latter was demonstrated to be partially bioavailable via oral administration in mice. Structure-activity relationship studies of both structural classes identified analogs with potent antitubercular activity, alone or in combination with meropenem. Transcriptional profiling revealed that oxidoreductases, MmpL family proteins, and a 27-kDa benzoquinone methyltransferase could be the targets of the *N*-arylindole potentiator. In conclusion, our compound-compound synthetic lethality screening revealed novel small molecules that were capable of potentiating the action of meropenem, presumably via inhibition of the innate resistance conferred by β -lactam accessory proteins. β -Lactam compound-compound synthetic lethality may be an alternative approach for drug-resistant tuberculosis.

KEYWORDS high-throughput screen, β -lactams, synthetic lethality, tuberculosis

Drug-resistant tuberculosis (TB) continues to be a major threat to global public health, accounting for 3.5% of new TB cases and 18% of previously treated TB cases (1). New drug development for TB is a time- and resource-consuming process. Over the past five decades, only two new TB drugs have been licensed for clinical use as part of combination therapy to address drug-resistant TB, and those drugs were associated with serious limitations (2). As a potential parallel approach to new drug development, repurposing existing antibiotics, such as clofazimine (3, 4), the trimethoprim-sulfamethoxazole pair (5), linezolid (6), and β -lactams (7–17), for drug-susceptible and drug-resistant TB therapy has shown some promise. Administration of amoxicillin-

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clavulanic acid, when added to a second-line TB regimen, successfully treated two patients with a drug-resistant form of TB (18). A 2-day early bactericidal activity (EBA) study showed minimal effects of this pair when used alone (19), but a 7-day EBA study showed results comparable to those of ofloxacin, although inferior to those of isoniazid (20). In particular, multidrug-resistant (MDR) TB patients treated with imipenem, a member of the carbapenem class of β -lactams, responded well when the drug was combined with other first-line or second-line TB drugs (21). In a clinical study aimed at evaluating the therapeutic contribution of imipenem-clavulanate (IC), results showed that all 12 patients (4 with MDR TB and 8 with extensively drug-resistant [XDR] TB) who were treated with IC converted to sputum culture negativity and 7 of them were cured (22). In a large clinical study, however, addition of IC to an optimized background regimen (OBR) did not yield a significant difference from the OBR control (23). It is interesting that the combination of meropenem and clavulanic acid, in the presence of one or two active second-line TB drugs, was active against human XDR TB (24, 25) and was more effective than IC in a treatment study of patients with MDR/XDR TB (26). These data suggest that β -lactam antibiotics, when incorporated into an optimized regimen, may serve as useful additions to known, effective, first-line and second-line TB drugs.

The β -lactam antibiotics represent a large family of broad-spectrum antibiotics. Carbapenems are a subclass of β -lactam antibiotics that possess higher intrinsic resistance to the most prevalent β -lactamases (27). Representative members of this structural class include imipenem, meropenem, ertapenem, doripenem, biapenem, and the more recent, orally available faropenem and tebipenem (10, 28–30). The molecular targets of β -lactams are generally accepted to be penicillin-binding proteins (PBPs) such as the transpeptidases, a class of enzymes that catalyze the cross-linkages of transpeptides and thus the biosynthesis of the essential peptidoglycan of bacteria (31). *Mycobacterium tuberculosis* produces two types of transpeptidases, namely, the traditional D,D -(3,4)-transpeptidases and the L,D -(3)-transpeptidases (32–34). In the stationary growth phase, the peptidoglycan of *M. tuberculosis* predominantly contains linkages generated by L,D -transpeptidases (35). Meropenem is able to inhibit both D,D -transpeptidases and L,D -transpeptidases (33, 36, 37), suggesting its potential utility in TB chemotherapy. Meropenem, when combined with the β -lactamase inhibitor clavulanic acid, is effective against XDR TB *in vitro* (7). This finding implies that meropenem could be a drug candidate to be used in combination chemotherapy for drug-resistant TB.

Although β -lactams specifically target the PBPs, the molecular mechanisms that confer resistance are complex. In addition to mutations in the target PBPs (31, 38, 39), efflux pumps (including multidrug efflux pumps) have been identified to play a role in β -lactam resistance (38–42). In our recent gene-compound synthetic lethality studies, we found that ABC transporters, the putative lipoprotein LprQ, a putative acyl transferase (Rv1565c), transcriptional regulators (including the two-component system RegX3), and a polyketide synthase (PpsC) also played roles in β -lactam resistance (38). Bearing in mind the complex nature of resistance mechanisms for β -lactams, in the current study we screened a small-molecule library using a compound-compound synthetic lethality approach, in the presence of a subinhibitory concentration of meropenem. Here we report the *in vitro* and *in vivo* characterization of this compound-compound synergy, and we characterize the bacterial transcription response network upon treatment with a representative compound.

RESULTS

High-throughput synthetic lethality screen. Our high-throughput screen (HTS) was set up and validated using the concentration-response curves for meropenem and clavulanic acid alone and in combination (see Fig. S1 in the supplemental material). The 50% inhibitory concentration (IC_{50}) value for meropenem alone was 8 μ g/ml, whereas clavulanic acid alone was inactive even up to 100 μ g/ml. However, 50% inhibition of bacterial growth was achieved with the combination of 0.2 μ g/ml of clavulanic acid and

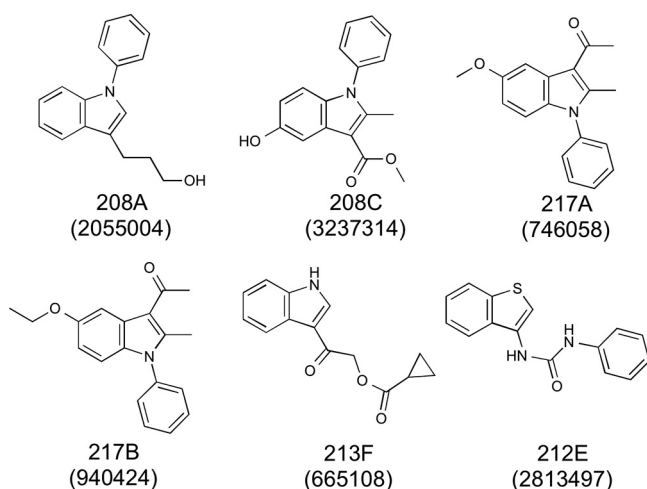


FIG 1 Structures of hit compounds. PubChem CID numbers are indicated in parentheses.

0.2 $\mu\text{g/ml}$ of meropenem. Based on these data, we used a meropenem concentration of 2 $\mu\text{g/ml}$ in the synthetic lethality screen, which barely inhibited *M. tuberculosis* growth, allowing evaluation of synergistic effects with small-molecule potentiators. Initial screening for compounds that were synthetically lethal in the presence of meropenem covered 318,012 small molecules of the NIH Molecular Libraries Small Molecule Repository (MLSMR) at Southern Research Institute (Birmingham, AL). Up to 876 molecules demonstrated preferential inhibition of *M. tuberculosis* growth, and rescreening in concentration-response analyses resulted in 363 noncytotoxic (72-h growth of Vero E6 cells) confirmed hits. Hit verification and confirmation were followed up, which resulted in six validated compound hits. Of the five indole derivatives, four have aryl substitutions on the indole nitrogen, while the benzothiophene has a urea moiety at the 3-position. (Fig. 1).

In vitro verification. Meropenem MICs, with or without the presence of 2 $\mu\text{g/ml}$ of each of the compounds, were determined for *M. tuberculosis* H37Rv. The MIC of meropenem alone was 16 $\mu\text{g/ml}$. Addition of 2 $\mu\text{g/ml}$ of three *N*-arylindoles and the benzothiophene lowered the MIC of meropenem from 16 $\mu\text{g/ml}$ to 8 $\mu\text{g/ml}$ (217A and 217B), 4 $\mu\text{g/ml}$ (213F), or 0.75 $\mu\text{g/ml}$ (212E) (Fig. 2A). No lowering of the MIC values was seen for the remaining two indoles, 208A and 208C. There was no observable inhibitory effect at 2 $\mu\text{g/ml}$ for each compound alone. In a reverse configuration, the MICs of each of the six compounds, with or without 2 $\mu\text{g/ml}$ of meropenem, were also analyzed. While two compounds had no (208C) or minimal (208A) synergistic effect with meropenem, addition of 2 $\mu\text{g/ml}$ of meropenem to the compounds significantly decreased their MICs as much as 16-fold (217B and 213F) or 32-fold (217A and 212E) (Fig. 2B). Again, no inhibitory effect was observed for meropenem alone at 2 $\mu\text{g/ml}$.

Structure-activity relationship studies. Encouraged by the ability of some *N*-arylindoles (217A, 217B, and 213F) and the benzothiophene 212E as meropenem sensitizers, we synthesized 68 indole analogs and screened them in the presence of a subinhibitory concentration of meropenem (Table S1). All 68 compounds have been assigned PubChem compound identifier (CID) numbers and respective BioAssay assay identifier (AID) numbers (PubChem BioAssay AID no. 434955, 485340, 504702, 504703, and 504713). The majority of the 68 indoles are 5-substituted with a hydroxyl or alkoxy group and 3-substituted with a methylketone or ethyl ester. The *N*-arylation on the indole generally did not result in more potent anti-TB compounds. In addition to the three hit indoles, 217A, 217B, and 213F, two more indoles (entries 4 and 5 in Table 1) were identified to be synthetically lethal for *M. tuberculosis*, showing 16- or 4-fold MIC reduction in the presence of 4 $\mu\text{g/ml}$ of meropenem (Table 1). As controls, rifampin and isoniazid were included in the screen, and no synergy was observed when they were

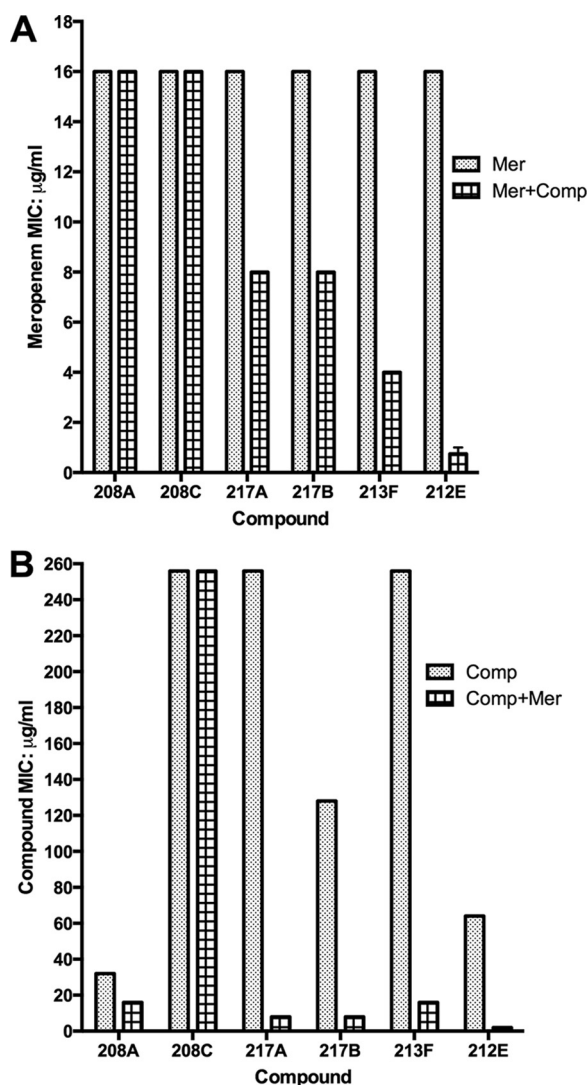
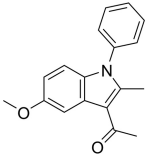
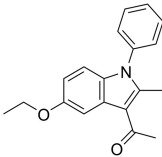
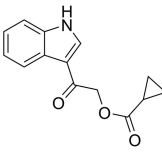
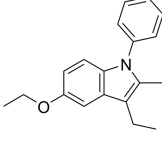
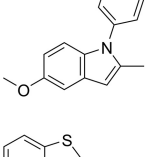
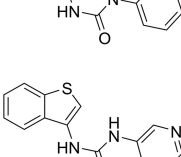
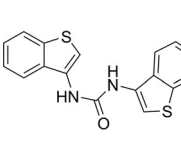
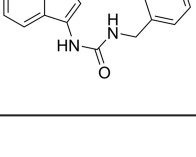
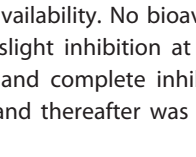


FIG 2 Synthetic lethality. (A) MICs of meropenem (Mer) in the presence or absence of 2 µg/ml of each of the six hit compounds (Comp). (B) MICs of hit compounds in the presence or absence of 2 µg/ml of meropenem. Data are shown as averages of three independent experiments.

combined with 4 µg/ml of meropenem (Table S1). Interestingly, we also discovered that five indole compounds (entries 1 to 5 in Table 2) were moderately active against *M. tuberculosis* in the absence of meropenem, showing MIC values of 4 or 8 µg/ml (Table 2). Similarly, we screened 39 benzothioephene and/or urea derivatives (Table S2) and found that four compounds showed synthetic lethality in the presence of 4 µg/ml of meropenem, with MIC reductions of 8- to 32-fold (Table 1). We also found that three urea compounds were active against *M. tuberculosis* in the absence of meropenem, showing MICs of 2 µg/ml (Table 2). Structurally, the urea derivatives resemble AU1235, an adamantyl urea derivative previously reported to be an MmpL3 inhibitor (43).

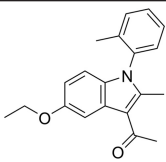
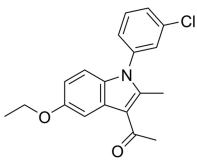
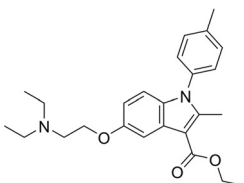
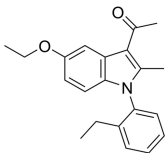
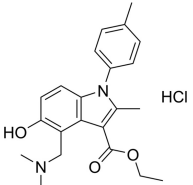
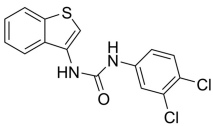
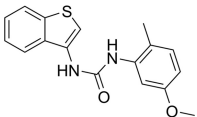
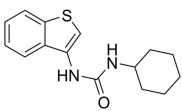
Serum inhibition titration assay for bioavailability. The mouse serum inhibition titration assay is a simple method to determine functional oral bioavailability of a compound together with any potential active metabolites. We administered 300 mg/kg of compound 217A to mice and tested the serum for *M. tuberculosis* growth inhibition in the presence of 2 µg/ml of meropenem. Compound 217A was orally bioavailable in mice and showed partial inhibition at 4- to 16-fold serum dilutions at the 15- and 30-minute postdosage time points (Fig. S2). Compound 217B did not show bioavailability at any time point or at either dosing level. At the given dosing level (300 mg/kg)

TABLE 1 Compounds showing synthetic lethality with meropenem against *M. tuberculosis*

Entry	PubChem CID no.	Class	Structure	MIC ($\mu\text{g/ml}$)	MIC with meropenem ($\mu\text{g/ml}$)	Fold change
1	746058	Indole		≥ 256	8	32
2	940424	Indole		≥ 128	8	16
3	665108	Indole		≥ 256	16	16
4	49842883	Indole		32	2	16
5	2329649	Indole		32	8	4
6	2813497	Urea		64	2	32
7	66545921	Urea		64	4	16
8	13182769	Urea		8	1	8
9	66545927	Urea		64	8	8

and time point (30 min), the benzothioephene compound 212E showed meropenem-concentration-dependent bioavailability. No bioavailability was observed when meropenem was at 0 or 2 $\mu\text{g/ml}$, slight inhibition at 8-fold dilution was observed when meropenem was at 4 $\mu\text{g/ml}$, and complete inhibition at 8-fold dilution and partial inhibition at 16-fold dilution and thereafter was observed when meropenem was at 8 $\mu\text{g/ml}$ (Fig. S2).

TABLE 2 Compounds active against *M. tuberculosis* without showing synthetic lethality with meropenem

Entry	PubChem CID no.	Class	Structure	MIC ($\mu\text{g/ml}$)
1	949476	Indole		8
2	804894	Indole		4
3	53308696	Indole		8
4	53308700	Indole		8
5	2882229	Indole		8
6	66545934	Urea		2
7	66545916	Urea		4
8	66545929	Urea		2

***N*-Arylindole treatment response networks assessed by RNA-Seq.** To seek the molecular target of the *N*-arylindole, we characterized the *M. tuberculosis* gene transcriptional responses on exposure to compound 217A, using whole-transcriptome shotgun sequencing (RNA-Seq) (Table S3). Differential expression was analyzed, and results are shown in Fig. 3 and Table S4. Thirty-three genes were upregulated at least

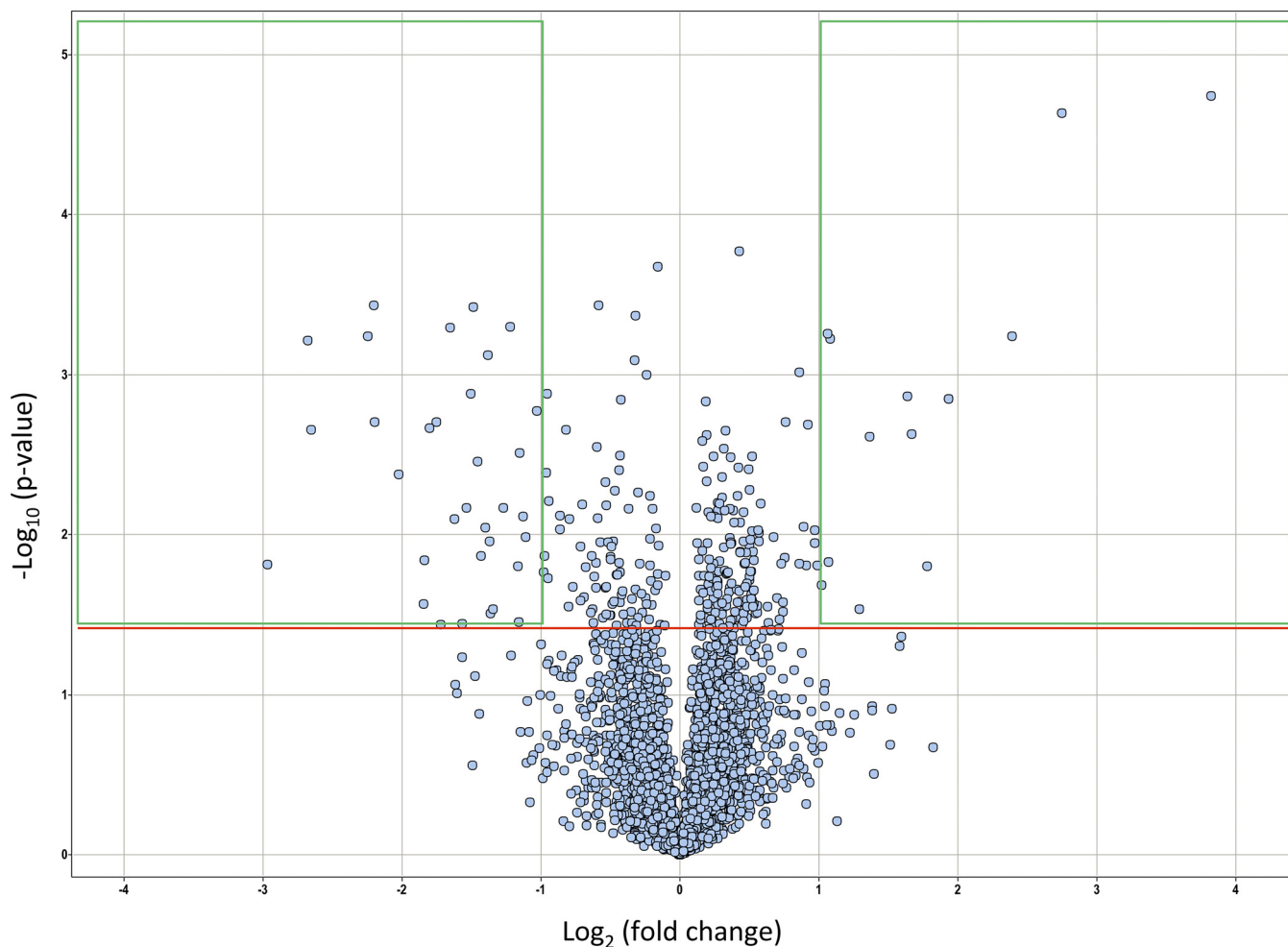


FIG 3 Volcano plot of differentially expressed genes after *N*-arylidole (compound 217A) treatment. The horizontal red line indicates a *P* value of 0.05. Green-framed boxes indicate fold changes of at least 2, either downregulated (left) or upregulated (right).

2-fold, and 15 showed statistical significance ($P < 0.05$). Fifty genes were downregulated at least 2-fold, with 34 showing significance ($P < 0.05$) (Table S4). Data analysis revealed that four of the resistance-nodulation-cell-division (RND) superfamily genes (*mmpS4*, *mmpS5*, *mmpL5*, and *mmpL6*) were significantly upregulated on exposure to compound 217A. Interestingly, the efflux pump regulator Rv0678 was significantly upregulated along with its substrates (*mmpS5* and *mmpL5*) (Table S4), suggesting a possible mechanism for β -lactam removal or, alternatively, delivery of peptidoglycan substrates that are depleted due to β -lactam action. The organic substance responder Rv0077c (probable oxidoreductase), along with the immediately downstream membrane protein Rv0076c, was highly upregulated; the 27-kDa putative benzoquinone methyltransferase Rv0560c and Mce3C, which plays a role in virulence, detoxification, and adaptation, were also significantly upregulated. Other upregulated genes included those for a transcriptional regulator (Rv0452), an exported protein (Rv0320), and conserved hypothetical proteins (Rv1434, Rv2003c, and Rv2722).

Multiple categories of genes were repressed or downregulated by *N*-arylidole treatment. A clear pattern shows that four proline-rich proteins (Rv3413c, Rv1435c, Mtc28, and Rv0312) were significantly downregulated and, interestingly, three invasion-associated genes (Rv1478, Rv1477, and Rv1566c) were also significantly downregulated. A few enzymes of metabolic pathways, such as a nitrite reductase (NirD), an oxidoreductase (Rv0063), an integrase/recombinase (XerC), an esterase (LipL), an acyl-coenzyme A dehydrogenase (FadE5), an acid phosphatase (SapM), a L-lactate dehydro-

genase (LldD1), a possible chitinase (Rv1987), and a lipase (LipU), were also downregulated. Additionally, transcription regulator WhiB2, the alternative RNA polymerase sigma factor SigD, probable cation transporter P-type ATPase D (CtpD), and the antigen 85C (FbpC) were downregulated, as were some conserved hypothetical proteins (Table S4). Apparently, treatment of *M. tuberculosis* bacilli with the β -lactam synergistic partner 217A would be stressful for the bacilli, thus promoting the expression of stress response genes, including those encoding efflux pumps, and reducing the expression of energy transport and metabolism genes, such as that encoding the alternative sigma factor SigD.

In silico analysis of differentially expressed genes, using either KOBAS (44) or PANTHER (45) tools, did not reveal significantly enriched metabolic pathways. However, gene ontology enrichment analysis of those genes indicated significant changes in external encapsulating structure (cell wall) components (Fig. S3), showing spatial proximity to the site of action of the synthetic lethality partner meropenem. In addition, biological processes such as responses to hypoxic nitrosative stress and host immune responses and cell growth were significantly altered.

DISCUSSION

Our previous HTS of the NIH MLSMR for activity on *M. tuberculosis* growth *in vitro* identified several novel chemotypes, including the candidate probe (CID no. 2792221), which showed a submicromolar MIC and nondetectable toxicity (46). However, the bifurcated molecular metabolic pathways of *M. tuberculosis* justify the search for synthetic lethality partners of known targets for approved cognate antibiotics, such as L,D - or D,D -transpeptidases and β -lactams (38). β -Lactams are not considered first-line TB drugs because of their suboptimal activity against *M. tuberculosis*. However, when combined with the β -lactamase inhibitor clavulanic acid, meropenem is active not only on susceptible *M. tuberculosis* but also on drug-resistant *M. tuberculosis* (7). Indeed, Payen and colleagues treated six patients with XDR TB using meropenem and clavulanate as a salvage regimen and successfully cured one patient; five of the six patients achieved sputum culture conversion (24, 47). Although meropenem inhibits both classic D,D -transpeptidases and nonclassic L,D -transpeptidases of *M. tuberculosis* (33, 36, 37, 48) and meropenem in combination with clavulanic acid was bactericidal to XDR TB *in vitro* (7), the combination of meropenem and clavulanic acid had no effect in a chronic mouse infection model and had marginal effects in an acute mouse infection model (9). Recent studies have shown that new, orally bioavailable penems (tebipenem and faropenem) (10, 30) and cephalosporins (49, 50) are also active on *M. tuberculosis*, suggesting a potential role for this class of drugs in outpatient treatment of TB, and the compounds in the current study may serve as potentiators for this class of drugs.

We found that RND family proteins were actively involved in "detoxification" of the *N*-arylindole compound 217A, as reflected by significant upregulation of the four genes in the family (*mmpS4*, *mmpS5*, *mmpL5*, and *mmpL6*) (see Table S4 in the supplemental material). Two more *mmpL* genes (*mmpL4* and *mmpL11*) were upregulated significantly, based on *P* values, but fell short of the 2.0-fold change cutoff value (Table S3). Interestingly, two *mmp* genes (*mmpS3* and *mmpL3*) were significantly downregulated, based on *P* values (Table S3). The RND family proteins of *M. tuberculosis* have not been well characterized. The *mmpS5*-*mmpL5* operon appears to be active in *M. tuberculosis* and plays a role as an efflux pump in azole resistance (51), probable meropenem resistance (38), and the probable *N*-arylindole resistance in this study (Table S4). This operon is regulated by the downstream MarR-like regulator Rv0678 (52), which was also significantly upregulated in this study (Table S4). Proteins in this class generally show weak substrate specificity and thus are less likely to be the true targets of the compound in question.

The most upregulated gene after treatment with the *N*-arylindole 217A was Rv0077c, encoding a probable oxidoreductase. Its immediate downstream gene, Rv0076c, encoding a membrane protein, was also significantly upregulated (Table S4). While it is possible that Rv0077c is the target for 217A and plays a required role in

peptidoglycan biosynthesis, it is probable that Rv0077c participates in detoxification of 217A. In either case, these processes may take place on the cell wall, with Rv0077c and Rv0076c working cooperatively. The upregulation of the 27-kDa methylase with 217A treatment was interesting, because previous studies showed that this gene was induced by exposure to salicylate (53) or rifampin (54). Structurally, 217A, salicylate, and rifampin are unrelated, implying the 27-kDa methylase could be a universal chemical stress responder. Structural similarity between benzothiophene and AU1235 (43) implies that they may share a molecular target; however, we did not explore the molecular target for this chemotype. Further work will be required to detail the molecular mechanism for the synthetic lethality effect of *N*-arylindole or benzothiophene with meropenem. We note that the *N*-arylindoles and the benzothiophene urea classes are not β -lactams and thus are unlikely to be direct, orthosteric, β -lactamase inhibitors. This was reflected by the fact that expression of the β -lactamase-encoding *blaC* gene was not significantly changed after 217A treatment (Table S3). Similarly, the *N*-arylindole compound was likely not targeting Ldt_{MT2}, the major L,D-transpeptidase that generates the 3-3 cross-links of the peptidoglycan synthetic pathway (32), as the gene expression did not change significantly after treatment ($P = 0.168$) (Table S3). Minor changes (fold change of <2.0) were seen for analogs of Ldt_{MT2}, such as Rv0116c (fold change of 1.365), Rv0483 (fold change of -1.194), and Rv1433 (fold change of -1.966). Similarly, no significant changes were seen for the two annotated D,D-transpeptidases (Rv2911 and Rv3330), which is noteworthy because meropenem works on both D,D- and L,D-transpeptidases (33). This finding implies that a new mechanism of synergy between *N*-arylindoles and meropenem exists, which warrants further studies.

The proof of concept for compound-compound synthetic lethality in *M. tuberculosis* was clearly shown *in vitro* in this study. The nonoptimal *in vivo* bioavailability could be improved with medicinal chemistry approaches. The individually active *N*-arylindole and benzothiophene compounds identified in the structure-activity relationship (SAR) assay are interesting and should be further investigated for their drug development potentials.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* H37Rv was used in this study. BD Difco Middlebrook 7H9 broth with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.2% glycerol, and 0.05% Tween 80 was used for initial bacterial culturing. Synthetic lethality assays, including the HTS, verification, and the SAR screen were carried out in the same medium but without Tween 80.

High-throughput screen. The HTS was carried out in the presence or absence of 2 μ g/ml of meropenem. Compounds were screened at a concentration of 25 μ M, in a total volume of 50 μ l and in a 384-well plate format. The dimethyl sulfoxide (DMSO) content was 1%, and the inoculum was 2.5×10^3 to 5.0×10^3 CFU per well. Clavulanic acid (β -lactamase inhibitor) and amikacin at 2.5 μ g/ml were included on each microtiter plate as positive controls. Plates were incubated for 7 days. Detection was achieved by addition of 9 μ l of alamarBlue, and plates were read 24 h later with a plate reader (excitation wavelength, 530 nm; emission wavelength, 590 nm). A summary of the screening campaign can be found at PubChem AID no. 434999 (<http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=434999>).

Verification screen and MIC assay. Meropenem was prepared fresh daily. The assay was conducted in a 96-well plate format. The DMSO content was 1%, and the inoculum was 1.0×10^4 to 2.0×10^4 CFU per well. After 7 days of incubation, the alamarBlue assay was performed and plates were read with excitation and emission wavelengths of 544 and 590 nm, respectively. Amikacin at 2.5 μ g/ml was used as a positive control, and bacteria alone was used as a negative control. Medium was used as a blank control. System validation was reported previously (38).

MIC determination using the alamarBlue assay. The MIC was defined as the minimum concentration that inhibited at least 90% of the bacterial growth. Compound MICs were determined using the alamarBlue assay, in the presence or absence of 2 μ g/ml of meropenem. Meropenem MICs were determined similarly, in the presence or absence of 2 μ g/ml of each of the six hit compounds.

Serum inhibition titration assay for bioavailability. To test oral bioavailability, 6-week-old female BALB/c mice (Charles River Laboratories, Inc.) were used. For *N*-arylindole compounds 217A and 217B, a single dose at 100 or 300 mg/kg was delivered orally. At 15, 30, and 60 min after administration, the mice were sacrificed under isoflurane anesthesia and cardiac blood was collected. For benzothiophene compound 212E, a single dose at 300 mg/kg was administered orally and blood was collected 30 min after gavage. The vehicle (0.5% carboxymethyl cellulose) was used as a negative control, and the first-line TB drug isoniazid at 10 mg/kg was used as a positive control. Both positive- and negative-control blood samples were collected 15 min after administration. One mouse per dose level per time point was used. Blood was left undisturbed for 30 min at room temperature, to allow clot formation, and then was centrifuged at $1,000 \times g$ for 15 min. Serum was collected and processed for the alamarBlue assay. The

serum was serially 2-fold diluted using 96-well microplates, and 10^4 CFU of *M. tuberculosis* H37Rv was added to each well. After incubation for 7 days at 37°C, 32.5 μ l of alamarBlue working reagent (1 part 10% alamarBlue and 0.625 part 20% Tween 80) was added to each well; plates were read at excitation and emission wavelengths of 544 and 590 nm, respectively, after 16 to 18 h of incubation. For the 217A and 217B assays, final meropenem concentrations of 2 μ g/ml were added to all wells. For the 212E assays, final meropenem concentrations of 0, 2, 4, and 8 μ g/ml were added to wells. The Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine approved all animal procedures performed in this study (protocol no. MO19M98).

Structure-activity relationship assay. We synthesized 68 *N*-arylindole analogs and 39 benzothiophene analogs for SAR studies. All compounds have PubChem CID numbers (Tables 1 and 2; also see Tables S1 and S2 in the supplemental material). Purities of all final compounds were >95% (area percentage at 214 nm), as established by analytical high-performance liquid chromatography (HPLC) carried out with a Shimadzu HPLC system using a Waters C_{18} column. Inhibitory activities of analogs were characterized in the presence of 0 and 4 μ g/ml (for *N*-arylindole derivatives) or 0, 2, and 4 μ g/ml (for benzothiophene derivatives) of meropenem. Active compounds were identified as those showing at least 4-fold MIC decreases in the presence of meropenem. For detailed method descriptions, see the supplemental material.

RNA-Seq analysis. Laboratory strain H37Rv was grown to an optical density at 600 nm of 0.5. Compound 3 (KSC-09-217A) was added to the culture at a final concentration of 64 μ g/ml. DMSO at an equal concentration was added as a control. Cells were treated for 6 h at 37°C with shaking. Three biological replicates were carried out. Total RNA isolation, purification, quantification, and sequencing were performed as described previously (38). Fragments per kilobase of transcript per million mapped reads (FPKM) were generated using an in-house script, which internally uses BEDTools (55). FPKMs from 217A-treated and control cells were first subjected to quantile normalization and then transformed into \log_2 notation with the Partek Genomics Suite platform (Partek Inc., St. Louis, MO, USA). The distribution of the resulting values was examined across all cell samples, and genes for which the minimum \log_2 value was at least 2.0 were compared between 217A-treated and control cells, to determine the relative mRNA expression of these two classes. Since the relative gene expression values, expressed as \log_2 (ratio) or \log_2 (fold change), showed normal distribution, they were binned according to standard deviation from their median to provide cutoff thresholds for upregulation or downregulation. We carried out functional enrichment analyses, such as gene ontology and metabolic pathway analyses, by using the Term Enrichment Tool in AmiGO 2 (<http://amigo.geneontology.org/amigo>), KOBAS 2.0 (kobas.cbi.pku.edu.cn), and PANTHER (pantherdb.org). The parameter cutoff values were set for each of the tools used to obtain the significantly enriched gene ontology terms or pathways. A *P* value cutoff of 0.05 and a minimum number of gene products of 2 were used for the Term Enrichment Tool. A false discovery rate-corrected *P* value of 0.05 was used for KOBAS gene ontology and pathway enrichment analyses. A Bonferroni-corrected *P* value of 0.05 was used for the PANTHER statistical overrepresentation test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01319-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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