

Dual-Pharmacophore Pyrithione-Containing Cephalosporins Kill Both Replicating and Nonreplicating *Mycobacterium tuberculosis*

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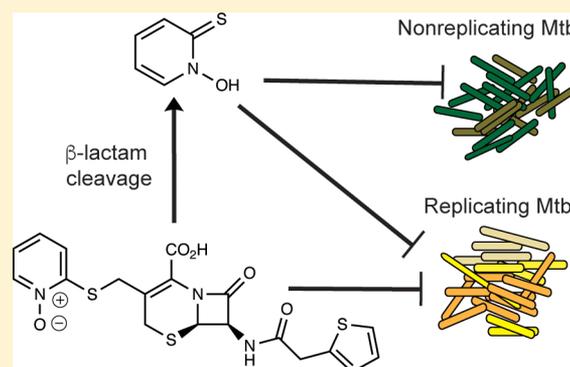
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Supporting Information

ABSTRACT: The historical view of β -lactams as ineffective antimycobacterials has given way to growing interest in the activity of this class against *Mycobacterium tuberculosis* (*Mtb*) in the presence of a β -lactamase inhibitor. However, most antimycobacterial β -lactams kill *Mtb* only or best when the bacilli are replicating. Here, a screen of 1904 β -lactams led to the identification of cephalosporins substituted with a pyrithione moiety at C3' that are active against *Mtb* under both replicating and nonreplicating conditions, neither activity requiring a β -lactamase inhibitor. Studies showed that activity against nonreplicating *Mtb* required the *in situ* release of the pyrithione, independent of the known class A β -lactamase, BlaC. In contrast, replicating *Mtb* could be killed both by released pyrithione and by the parent β -lactam. Thus, the antimycobacterial activity of pyrithione-containing cephalosporins arises from two mechanisms that kill mycobacteria in different metabolic states.

KEYWORDS: *Mycobacterium tuberculosis*, cephalosporin, pyrithione, β -lactamase, antimycobacterial



Mycobacterium tuberculosis (*Mtb*) is a leading infectious cause of death in the world.¹ Active tuberculosis (TB) is characterized by heterogeneous populations of mycobacteria in diverse microenvironments, ranging from cavities, necrotic granulomas, non-necrotic granulomas, and pneumonitic infiltrates to macrophages in otherwise healthy-appearing tissues.² In a given subject at a given time, some *Mtb* bacilli may be replicating and other bacilli may be replicating slowly or not at all. Non- or slowly replicating bacteria display

phenotypic tolerance to most antibiotics selected for their ability to kill replicating bacteria.^{3–5}

The cure of drug-sensitive TB involves prolonged treatment with isoniazid, rifampicin, pyrazinamide, and ethambutol. This may be necessary both to prevent selection for heritable antibiotic resistance and to overcome phenotypic tolerance.⁶ Except for pyrazinamide, known antimycobacterials are most

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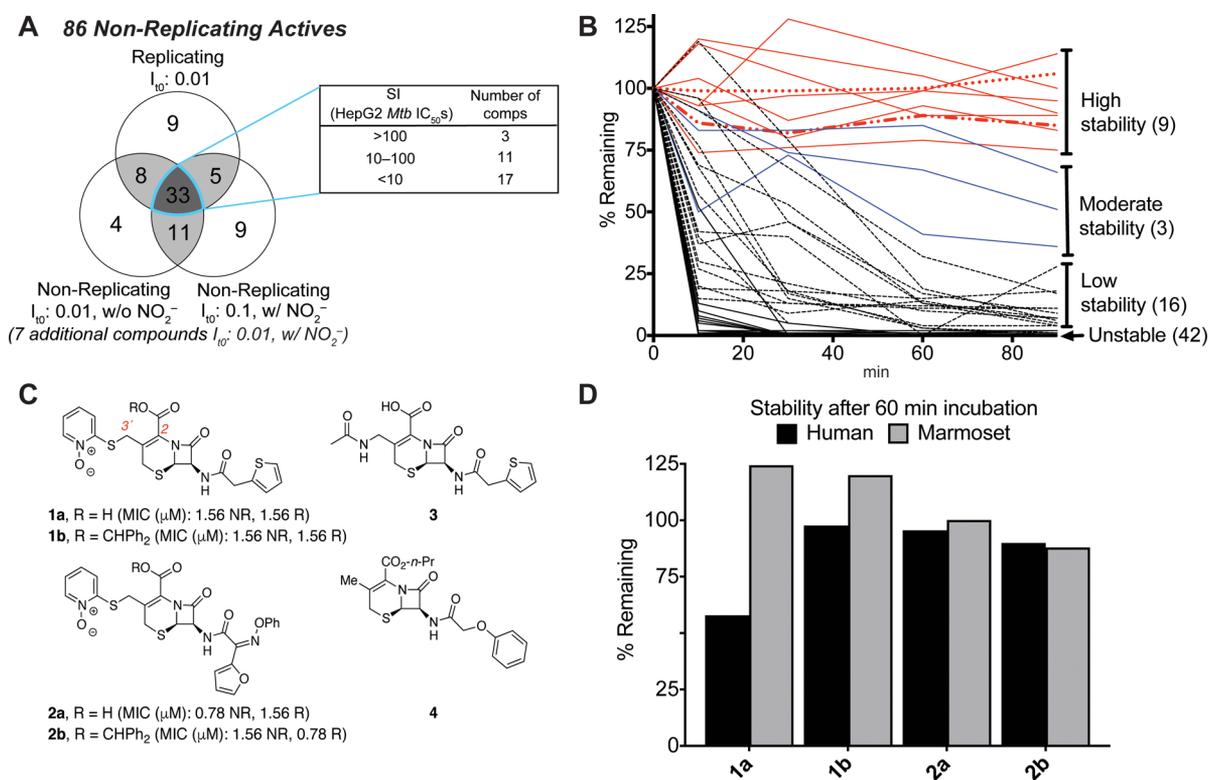


Figure 1. A whole cell screen resulted in the identification of 86 β -lactams active under NR conditions. (A) Analysis of Table S1 according to compound activity under replicating (R) or nonreplicating (NR) conditions with or without 0.5 mM NaNO_2 and with a starting inoculum of 0.1 or 0.01. Seven compounds were active only under NR conditions with NaNO_2 and low inoculum. (B) Stability of 70 out of the 86 active compounds in mouse plasma. Compound 1a is represented as a broken red line (— · —); compound 3 is represented as a dotted red line (····). This large-scale experiment was conducted once. (C) Structure and MIC₉₀ values of selected compounds. (D) Stability in human or marmoset plasma after a 60 min incubation at 37 °C of the dual-active compounds selected for further studies.

effective on replicating cells. In contrast, *Mtb* becomes phenotypically tolerant toward most TB drugs when subjected to hypoxia, nutrient starvation, acidity, reactive nitrogen intermediates, or combinations of these stresses.^{7–9} Given the rising global burden of TB resistance to front-line drugs and the toxicity, cost, and limited efficacy of second-line agents, a major goal for TB drug development is to find safe compounds that inhibit new targets in *Mtb*, thereby avoiding pre-existent resistance, and that kill *Mtb* in diverse metabolic and replicative states, including states associated with phenotypic tolerance to other agents.

β -Lactams are among the oldest antibiotics and have been so pervasive in infectious disease treatment that their historical absence from TB therapy is striking. Classic β -lactam classes have been considered ineffective against *Mtb* due to natural resistance of its predominant peptidoglycan transpeptidases, efflux pumps, production of a class A β -lactamase (BlaC), and limited permeability of its thick, waxy cell wall.^{10–13} The best-known targets of β -lactams are penicillin-binding proteins (PBPs) that catalyze the final D,D-transpeptidation steps in peptidoglycan biosynthesis. However, mycobacterial peptidoglycan formation relies largely on L,D-transpeptidases that are structurally distinct from PBPs,^{14,15} catalyze transpeptidation via an active site cysteine rather than serine,¹⁶ and are resistant to most β -lactams.¹⁷

In 1983, Cynamon et al. challenged the view that β -lactams could not be used to kill *Mtb* by demonstrating the antimycobacterial activity of amoxicillin combined with the β -lactamase inhibitor clavulanate.¹⁸ More recently, a clinical

trial indicated strong early bactericidal activity of meropenem in combination with amoxicillin and clavulanate.¹⁹ Most such studies have been carried out on replicating *Mtb*. However, Hugonnet et al. reported a ca. 1 order of magnitude reduction in colony-forming units (CFUs) after a 14-day exposure of hypoxic *Mtb* to meropenem plus clavulanate.²⁰ In 2016, using a combination of hypoxia, mild acidity, a flux of reactive nitrogen species, and a fatty acid carbon source to simulate conditions in the host and to impose nonreplication,²¹ we reported a new class of cephalosporins that cause multilog killing of *Mtb* but only when the *Mtb* is not replicating.²² The above results inspired us to seek β -lactams that were more broadly active against *Mtb*. Herein, we report the discovery of cephalosporins that kill both replicating and nonreplicating *Mtb* without dependence on clavulanate.

RESULTS AND DISCUSSION

High Throughput Screen for β -Lactams Active against Replicating and Nonreplicating *Mtb*. Working under the auspices of the Tres Cantos Open Lab Foundation at GlaxoSmithKline's (GSK's) Tres Cantos Campus, we selected for testing 1904 compounds representative of the >14 000 β -lactams in the GSK collection. This focused set included ca. 1330 cephalosporins, 370 monobactams, 150 penems, and 30 carbapenems. Seeking compounds that would be active under both replicating (R) and nonreplicating (NR) conditions, we tested the β -lactams against *Mtb* at 100 and 10 μM without a β -lactamase inhibitor under standard R culture conditions and three NR conditions that combined hypoxia,

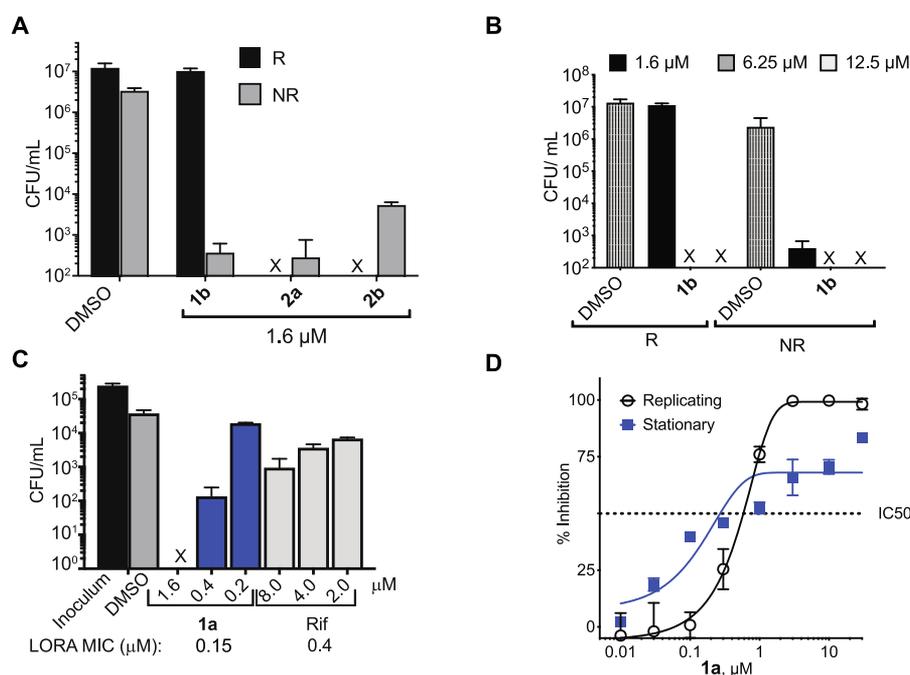


Figure 2. Activity of PYR-containing cephalosporins. (A) Quantification of the change in CFU/mL of **1b**, **2a**, and **2b** after 7 days of drug exposure. “X” indicates the CFU was below the limit of detection. (B) Concentration dependence of **1b** activity, indicating its cidal activity at higher concentrations. (C, D) NR activity of **1a** as assessed by the LORA and BCG stationary phase assays, respectively. Graphs represent the mean \pm SD of triplicate samples. Experiments were conducted ≥ 2 times.

mild acidity, and a fatty acid carbon source. In two of the NR conditions, we included a flux of reactive nitrogen species generated from nitrite at the low pH.^{4,23} Because the extent of killing by β -lactams is often inversely correlated with the initial bacterial concentration,^{24–26} we tested *Mtb* at a low initial inoculum (OD₅₈₀ of 0.01, where OD is optical density) in three of the four conditions and one NR condition at an OD₅₈₀ of 0.1 (Figure 1A and Table S1).

After eliminating compounds that were only active under R conditions, 86 compounds remained that had an IC₅₀ < 100 μ M in one or more of the three NR conditions, and 55 of these were also active against R cells (Figure 1A and Table S1). Of the 1904 β -lactams tested, 4.1% were active in at least two conditions tested, a high hit rate compared to the <1.5% hit rates reported in other whole cell *Mtb* screens (and noteworthy given the above-noted history of β -lactams in the *Mtb* area).^{23,27} Of these dual-active compounds, 33 were active against NR cells whether or not nitrite was included in the medium and whether or not the inoculum was high or low. Fourteen of these 33 most broadly active compounds had a selectivity index (SI) ≥ 10 when their cytotoxicity was tested against HepG2 human hepatoma cells (Figure 1A, inset table). Unfortunately, two of the broadly active compounds were not available for HepG2 assays, and the SI could not be determined. We tested the stability of 70 of the 86 active compounds in mouse plasma and eliminated 58 because of their plasma instability (Figure 1B).

Of the 9 dual-active compounds with high stability in mouse plasma, five were selected for the present study. The previously reported compound **1a**²⁸ was active under all four conditions tested. Three other compounds were structurally related to **1a** and shared its activity profile, while a representative inactive compound, **3**, was selected for comparison purposes. All four actives contained a pyrithione (PYR) heterocycle attached at C3' (Figure 1C). PYR, derived from the natural product

aspergillitic acid, has a long history in medicinal chemistry, having been reported to have antimicrobial activity against *A. niger*, *C. neoformans*, *M. bovis* Bacille Calmette-Guérin (BCG), *S. aureus*, and *K. pneumoniae*, among other fungi and bacteria.^{29,30} It has recently been studied, as the copper complex, in *Mtb*.³¹

We also noted that compounds **1a** and **2a** bear a carboxylic acid at C2, as is typical of antibacterial cephalosporins, while **1b** and **2b** have a diphenylmethane ester at this position. These β -lactams were stable in marmoset and human plasma (Figure 1D), although compound **1a** was slightly less stable in human plasma. All four compounds retained activity over at least 72 h at 37 °C in both acidified nitrite-containing NR or R media (data shown for **2a** only; Figure S1A,B).

The cephalosporins were subjected to further anti-*Mtb* characterization under NR conditions that included nitrite and used an initial inoculum of OD₅₈₀ of 0.01. Activity was not limited to growth inhibition but represented cidal activity. After only 7 days of exposure at 1.6 μ M, **2a** and **2b** reduced the CFU of R *Mtb* by 5 orders of magnitude and of NR *Mtb* by >3 orders of magnitude (Figure 2A). Compound **1b** required a higher concentration, 6.26 μ M, to reduce the CFU of both R and NR *Mtb* by 5 orders of magnitude (i.e., below the limit of detection) (Figure 2B).

Cephalosporin **1a** was assessed in three different models of nonreplication. The low oxygen recovery assay (LORA)³² assesses activity of bacteria placed in a state of phenotypic tolerance to most antimycobacterial agents by hypoxic conditions. In this assay, **1a** reduced the *Mtb* CFU/mL by at least 4 orders of magnitude at 1.6 μ M, retaining its potency in contrast to the rifampicin control, which lost activity under similar conditions (Figure 2C). In the *M. bovis* BCG stationary phase model, cells were grown in nutrient rich media for 40 days. Stationary phase BCG cells were frozen for storage and then resuspended in PBS to test for compound sensitivity.

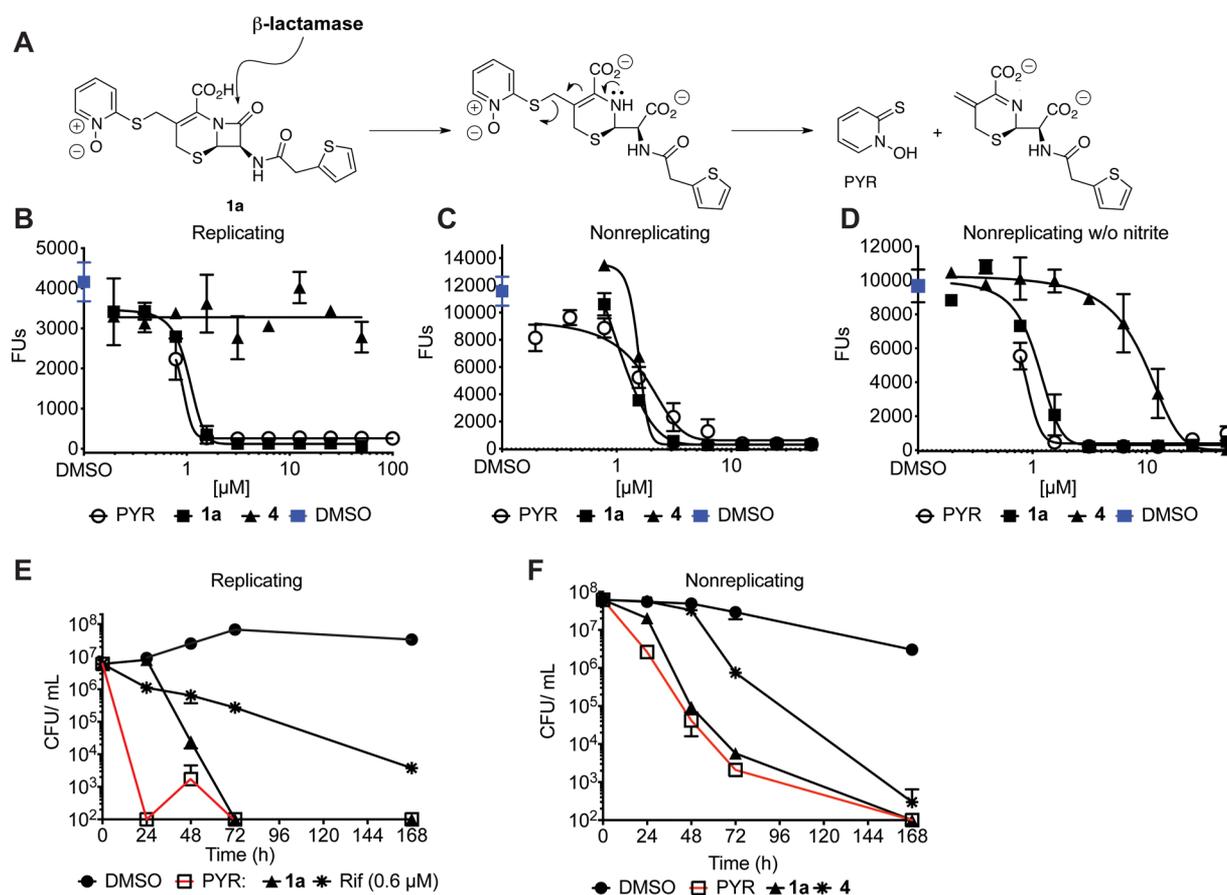


Figure 3. Studies of the mode of action of the PYR containing-cephalosporins. (A) Hypothetical release of PYR from the cephalosporin backbone following β -lactamase cleavage. (B–D) CARAs were used to compare the activity profile of 1a, 4, and PYR under R conditions and NR conditions with and without nitrite. (E, F) CFU/mL reduction over time when cells are exposed to 1.5 \times CARA_{MBC} concentrations of the 1a and PYR sodium salt under R and NR conditions as compared to rifampicin under R conditions or 4 under NR conditions. Results are means \pm SD of triplicates. Experiments were conducted at least twice.

Here, 1a partially lost activity (but not completely) compared to R cells (Figure 2D). Additional experiments confirmed that NR activity was not due to carryover of compound from the R to the NR phase of the assay. This was accomplished using the charcoal-agar resazurin assay (CARA),⁷ which controls for carryover of compound by absorbing residual compound found in the NR media. In this assay, the action of 1a and 2a was nitrite independent (Figure S2A,B), unlike a representative NR-selective cephalosporin 4²² (Figure S2C), and was not due to the cephalosporins inhibiting *Mtb*'s ability to transition from R to NR conditions (Figure S2D).

Role and Mechanisms of PYR Release. An established feature of cephalosporins is their ability to release a C3' nucleofuge upon opening of the β -lactam ring (Figure 3A). First noted by Abraham and co-workers in 1965^{33–35} and mechanistically examined biochemically^{36–38} and theoretically³⁹ some decades later,^{33,36–38} this concept has been extensively applied to anti-infective agent design,^{42–51} including recently against *Mtb*.^{52,53} This aspect of cephalosporin chemistry has also been leveraged to deliver other classes of bioactive molecules, such as antibody conjugates against cancer^{54–59} or cephalosporins bearing chromophores for β -lactamase detection.^{60–63} Most relevant to the present work is the incorporation of a PYR moiety to cephalosporin derivatives by O'Callaghan and co-workers⁴⁰ and Greenwood and O'Grady⁴¹ in 1976. To investigate the contribution of PYR

to the antimycobacterial activity of the cephalosporins, we tested the activity of PYR sodium salt as a function of concentration and time. We found that PYR was equipotent on a molar basis with cephalosporin 1a under both R and NR conditions and its activity did not depend on the inclusion of nitrite in the assay (Figure 3B–D). In terms of the rate of killing, at 2.5 μ M (1.5 \times the CARA_{MBC} for R *Mtb*, where MBC is minimum bactericidal concentration), PYR reduced the CFU of R *Mtb* by >5 orders of magnitude within 24 h. In contrast, an equimolar concentration of 1a took 72 h to reduce the CFU/mL of R *Mtb* to the same extent (Figure 3E). However, under NR conditions, both compounds at 2.5 μ M killed *Mtb* at the same rate and to the same extent (Figure 3F). For comparison, killing was much slower when rifampicin was tested against R *Mtb* (Figure 3E) or 4 was tested against NR *Mtb* at their 1.5 \times CARA_{MBC} concentrations (Figure 3F).

The antibacterial activity of PYR has been proposed to arise from its ability to chelate metals^{64,65} or disrupt disulfide bonds in certain proteins.^{66,67} For PYR to act by such mechanisms, it must first be liberated from the cephalosporin molecule.⁵² The foregoing results suggested that PYR release could account quantitatively for the activity of the dual-active cephalosporins under both R and NR conditions but could account kinetically for killing only if PYR is released slowly under R conditions and quickly under NR conditions. In previous studies, O'Callaghan et al. and Zaengle-Barone et al. ascribed the

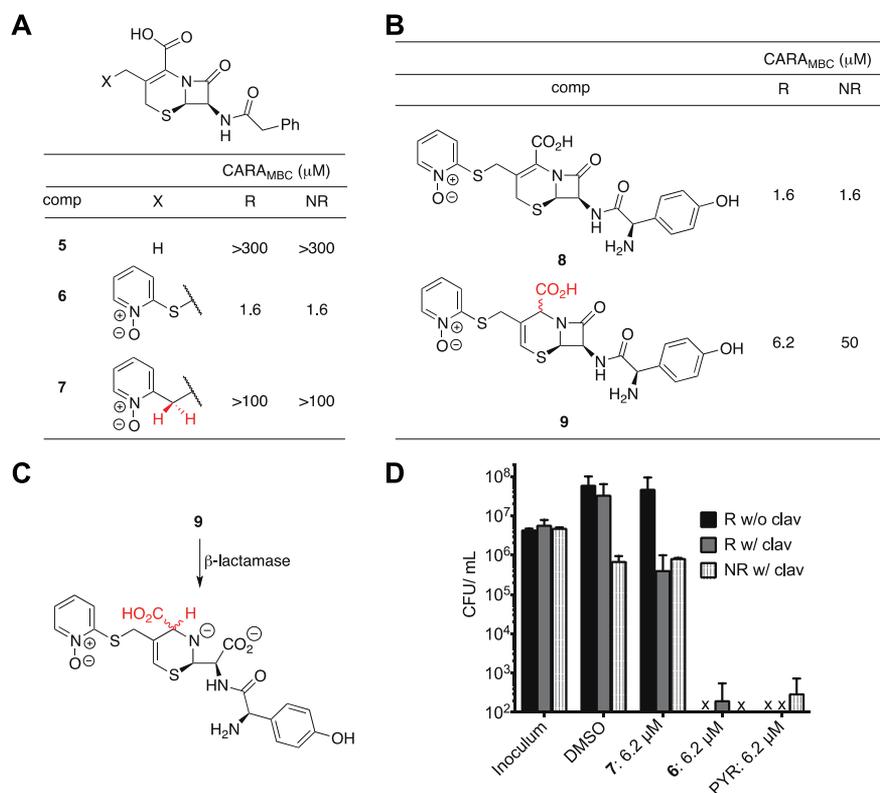


Figure 4. (A) Comparison of a compound able to release PYR (6) with analogues lacking a PYR moiety (5 and 7). Activity was measured as CARAMBC. (B) Comparison of a compound able to release PYR (8) with an analogue unable to do so (9). (C) Ring-opened form of compound 9. (D) CFU/mL reduction after 7 days of compound 6 and 7 exposure under R conditions without 25 μM clavulanate (black bar) or with clavulanate (gray bar) or NR conditions with clavulanate (striped bar). CARAMBC represents the concentrations at which compounds reduced the fluorescent signal to or below 1% of the signal seen with 1% DMSO as a control. CFU/mL reduction experiments were conducted in triplicate, and bars represent mean \pm SD.

activity of a pyrithione-containing cephalosporin against *E. coli* in part or completely to the expulsion and subsequent activity of the pyrithione.^{40,65}

A series of analogues was synthesized to address the necessity of PYR ejection for activity (Figure 4). Compound 5 was previously shown to lack activity against *Mtb*,²² but the analogue containing PYR at C3' is active under both R and NR conditions (Figure 4A). This is not surprising insofar as 6 differs from 1a by only the replacement of a side-chain thiophene with a phenyl group. Compound 6 was also recently reported by Zaengle-Barone et al. to be potently active against *E. coli* strains, particularly when the organisms had low levels of β -lactamases.⁶⁵ To test the role of PYR release, we prepared and tested the carbon-linked analogue 7, which was, on its own, inactive against *Mtb* under both R and NR conditions. However, with the addition of clavulanate, ca. 99% of the *Mtb* was killed in R, but not NR, assays. Similarly, meropenem in combination with clavulanic acid has been reported to kill 2 orders of magnitude of replicating *Mtb* cells.⁶⁸ This highlights the importance of activity of the parent cephalosporin backbone in situations where the bacterial expression of β -lactamase is low. Additional evidence was obtained by comparing the activity of the known⁴⁰ compound 8 with its $\Delta^{3,4}$ isomer 9, which was obtained as a byproduct of deprotecting the *N*-Boc precursor of 8 (compound 9 is a single isomer of unknown stereochemistry at C2; see the Supporting Information for details).⁶⁹ While the former can easily open and eject a C3' leaving group analogously to the mechanism in Figure 3A, 9 cannot because the nitrogen atom

revealed by the β -lactam opening is insulated from the leaving thiol (Figure 4C). The addition of 25 μM clavulanate partially restored activity to 7 under R but not NR conditions (Figure 4D).

Taken together, the above experiments suggest substantial activity against *Mtb* under R conditions in the presence of clavulanate independent of PYR release. Although this may represent a classic β -lactam mechanism entailing the inhibition of the final transpeptidation step in peptidoglycan synthesis, we have not yet done any experiments that inform this point and alternative targets are possible. In the case of compound 9, we also cannot exclude the possibility that the remaining activity arises from isomerization to 8 in the course of the experiment. However, since this is not possible for carbon-linked analogue 7, which also retains clavulanate-dependent activity against R *Mtb*, we conclude that direct action of the β -lactam is likely a contributor to anti-*Mtb* activity in R organisms.

As noted earlier, the PYR-containing cephalosporins retained activity after incubating in cell-free medium. However, this could be due to either survival of the parent β -lactam over the time course of the experiment or persistence of released PYR in the media. To address this, we confirmed that PYR is not released from 1a or a simple alkylated PYR analogue (2-(carboxymethylthio)pyridine-*N*-oxide, 10) in cell-free medium using an assay that detects the formation of an Fe^{3+} -PYR complex visually or by absorbance at 600 nm (Figure S3).⁷⁰ In contrast, we detected PYR in the medium of R or NR *Mtb* exposed to PYR-releasing 1a or 6 but not 10 or 7 (Figure 5). The amount of PYR detected in the medium of *Mtb* cultures

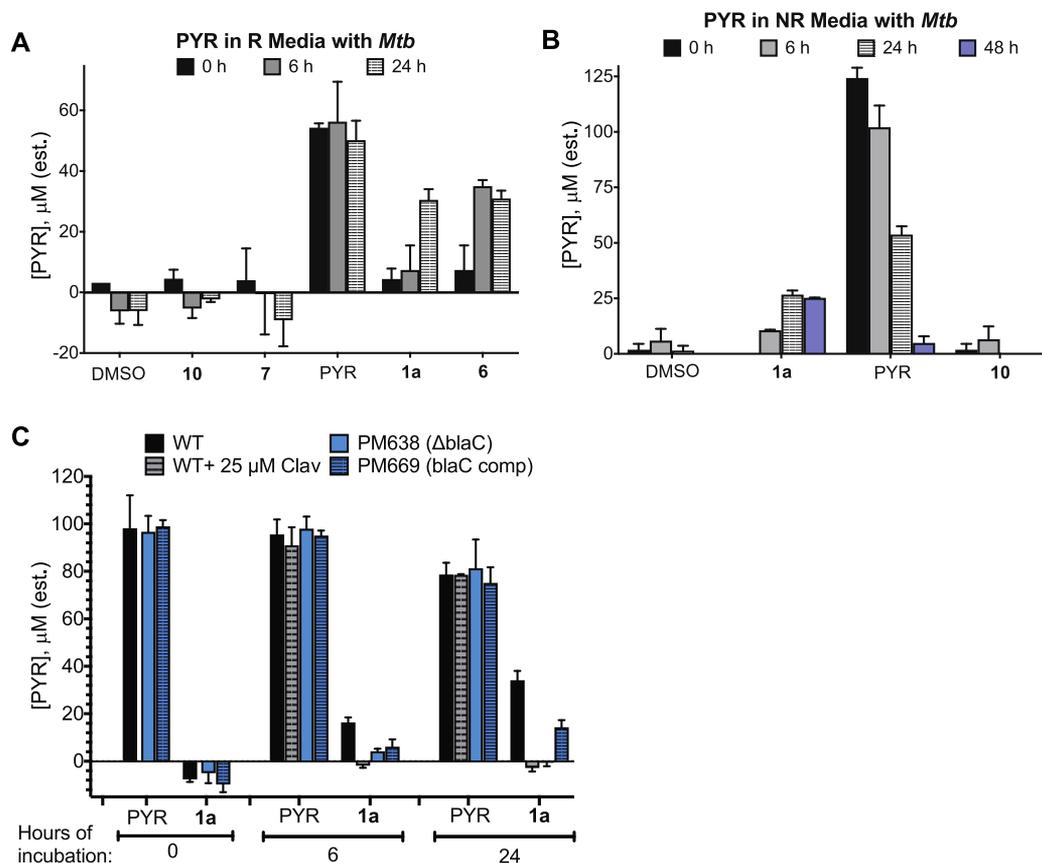


Figure 5. PYR found in media after incubation with whole cells. (A) Estimated levels of PYR in R medium after 6 or 24 h in the presence of an estimated starting 10^6 CFU and an initial compound concentration of $50 \mu M$. (B) Estimated levels of PYR in NR medium after 6, 24, and 48 h in the presence of an estimated starting 10^6 CFU and an initial compound concentration of $100 \mu M$. (C) Estimated levels of PYR release over 24 h in R conditions by the $\Delta blaC$ strain or complemented strain or WT *Mtb* cells alone or in the presence of $25 \mu M$ clavulanic acid. Results are means \pm SD of triplicates.

was about one-half the starting concentration of the cephalosporin under R conditions and one-quarter under NR conditions. However, while free PYR was stable over at least 24 h in the medium used for R conditions, it largely disappeared over 48 h from the medium used for NR conditions. We did not determine the fate of PYR in these experiments; it might disappear due to oxidative dimerization or conjugation. The proportion of PYR detected by the assay is the proportion remaining in the media or released by the bacterial cell, and thus, the amount detected over longer time points does not reflect PYR taken up by cells, released inside the cells, or degraded.

BlaC, a chromosomally encoded class A serine β -lactamase, is *Mtb*'s only functionally defined β -lactamase.⁷¹ In previous work, it was shown that deletion of the gene encoding BlaC and/or addition of clavulanate, a BlaC inhibitor, rendered *Mtb* susceptible to the cephalosporin cefoxitin, multiple penicillins such as piperacillin and mezlocillin, and the carbapenems imipenem⁷² and meropenem.²⁰ Other studies showed that *Mtb* can use BlaC to release a fluorophore or quencher from the C3' of a cephalosporin within *Mtb*.^{73,74} In accordance with these studies, the levels of PYR in the media were undetectable when WT cells were coinoculated with 1a and clavulanic acid or when BlaC was knocked out (Figure 5C). We verified the ability of BlaC to release PYR from the cephalosporin backbone by purifying a truncated, soluble form of BlaC as described.⁷¹ Within 10 min of incubation of $100 \mu M$ β -lactam

with $1 \mu M$ BlaC at $37^\circ C$, PYR was quantitatively released from the cephalosporins 1a, 6, and 8 but, as expected, not from 7 or 9 (Figure 6A). When 1a and cephalothin, which bears an acetoxy group at C3' (see Table 1 for the structure), were incubated with BlaC, the UV-visible absorption spectrum of the solution containing 1a converted to that of PYR while the spectrum of that containing cephalothin did not change (Figure 6B).

Thus, both *Mtb* and recombinant BlaC could release PYR from a PYR-containing cephalosporin. However, the mycobactericidal activity of these PYR-containing cephalosporins was unaffected by clavulanate at concentrations that inhibit BlaC within *Mtb* (Table 1). Moreover, the *Mtb* $\Delta blaC$ strain showed no change in 1a's $CARA_{MBC}$ under R conditions (Figure 6C) and, at the CFU/mL level, showed a greater sensitivity than WT to 1a (Figure S4A). No change in 1a activity was also noted under NR conditions (Figure S4B). In contrast, 3, the antimycobacterial activity of which is clavulanate dependent, was 10-fold more potent against the *Mtb* $\Delta blaC$ strain than against WT *Mtb* (Figure 6D) but had no gain in activity under NR conditions (Figure S4B). Complementation of the $\Delta blaC$ strain with the wild-type allele partly restored *Mtb*'s resistance to 3 in the absence of clavulanate (Figure 6D). Taken together, these observations suggest that the antimycobacterial activity of the compounds described here does not rely on either the action or inhibition of BlaC, *Mtb*'s functionally defined β -lactamase, despite the

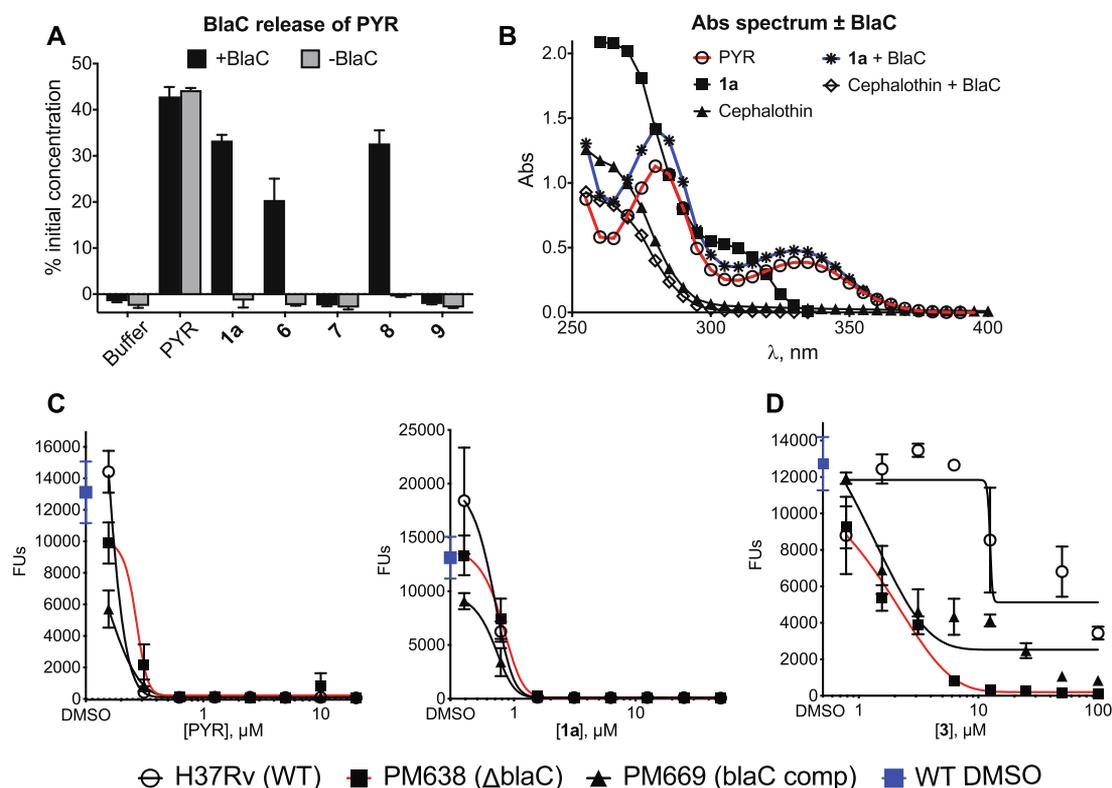
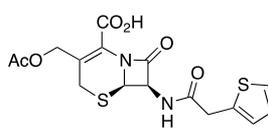
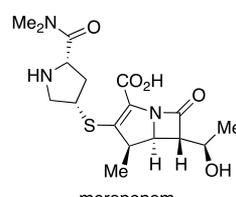


Figure 6. Role of BlaC in PYR release. (A) Estimated levels of PYR recovered after incubating with 1 μ M BlaC for 10 min represented as the percent of the initial concentration of parent compound. (B) The absorption spectrum of 100 μ M of cephalothin and 1a before and after 10 min of incubation with 1 μ M BlaC. (C) The efficacy of PYR and 1a in *M. tuberculosis* WT (open circles), BlaC KO (PM638, squares), and complemented strain (PM669, triangles). (D) The efficacy of clavulanate dependent 3 in *M. tuberculosis* WT (open circles), BlaC KO (PM638, squares), and complemented strain (PM669, triangles). Results are means \pm SD of triplicates.

Table 1. Clavulanate Dependence of Selected Cephalosporins and PYR

entry	compound	MBC (μ M)			
		R CARA	R CARA + 25 μ M Clav	NR CARA	NR CARA + 25 μ M Clav
1	1a	1.6	1.6	3.1	1.6
2	3	100	3.1	>100	>100
3	 cephalothin	>100	12.5	>100	>100
4	 meropenem	12.5	3.1	>100	>100
5	PYR	1.25	1.25	1.25	1.25

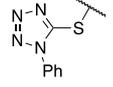
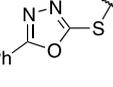
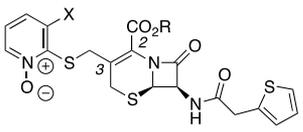
observation that the PYR–cephalosporins can be a substrate for BlaC. These results indicate that PYR can be released from the cephalosporins by an unknown, clavulanate-resistant, non-BlaC β -lactamase, an alternative covalent reaction with unknown cellular targets, or a nonenzymatic hydrolysis reaction. Voladri et al. presented evidence for the existence of a non-BlaC mycobacterial cephalosporinase,⁷⁵ and at least 12 proteins in *Mtb* besides BlaC are annotated to have sequence similarity to β -lactamases. Indeed, in the non-pathogenic *Mycobacterium smegmatis*, an appreciable loss of **1a** activity was detected in the double β -lactamases knockout but not the single knockout (Figure S4C). An alternative explanation would be that these cephalosporins have a second, non-PYR-dependent mechanism of action, most likely related to “normal” β -lactam reactivity as suggested by the activity profile of compound **7**.

Spectrum of Activity, Structure–Activity, and Preliminary Pharmacokinetics Studies. The hit cephalosporin **1a** was tested against 11 clinical isolates of *Mtb*. The MIC₉₀'s (where MIC is minimum inhibitory concentration) ranged from 0.8 to 1.9 μ M whether the isolates were drug-sensitive, monoresistant, or multidrug resistant (Table S2). In contrast, when selected PYR–cephalosporins were tested for activity against ESKAPE pathogens, methicillin-sensitive *S. aureus* was sensitive to the cephalosporins (MIC₉₀ range: 0.2–0.78 μ M), including the carbon-linked cephalosporin **7**, but not to PYR (MIC₉₀ 25 μ M) (Table S3) indicating that this activity is most likely due to the cephalosporin backbone rather than the PYR. The PYR-bearing cephalosporins (and PYR) were poorly active against *E. faecium*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* (MIC₉₀ 12.5–>100 μ M, Table S3). Consistent with these observations, we have now shown that outfitting selected cephalosporins with PYR payloads at C3' can confer relatively narrow bactericidal activity against *Mtb*. The released PYR may be solely responsible for bactericidal activity, although we cannot exclude other β -lactam mechanisms at this stage.

We briefly examined the structure–activity relationships of dual-active cephalosporins. As expected from the evidence that the PYR contributes to the activity of these cephalosporins against *Mtb*, replacement of the PYR moiety with alternative C3' moieties abolished activity against *Mtb* (Table 2, entries 1–3). In contrast, placing a PYR onto a compound previously shown²² to have only activity against NR *Mtb* conferred dual activity (entry 4). However, like other compounds containing an ester in that series, this material was unstable in mouse plasma. Two analogues containing substitution on C6 of the PYR were also potently active, especially in NR assays (entries 5 and 6). Finally, selected analogues were found to be nontoxic to mammalian cells (Table S4).

Although compound **1a** was stable in mouse and primate plasma (Figure 1D) and in mouse and human liver microsomes (Figure S5A), orally administered **1a** was cleared rapidly from the blood of mice after oral or parenteral administration (Figure S6A). In contrast, compound **8**, which was reported 40 years ago,⁷⁶ was both stable to microsomes of various animals and measurable in mouse plasma for more than 5 h after an oral dose of 20 mg per kg or even longer when administered by intraperitoneal (IP) dosing (Figure S6B). The bioavailability of **8** was 5.2% after oral gavage and 31.8% after intraperitoneal injection. At 150 mg/kg, compound **8** was well tolerated by mice for at least 24 h after oral administration.

Table 2. Activity of Additional Analogues

entry	compound	MIC ₉₀ (μ M)			
		R	NR		
		X	Ar		
1	11	SPh	Ph	74.0	>100
2	12		Ph	>100	>100
3	13		Ph	>100	>100
		X	R		
4	14	H	<i>n</i> -Pr	1.14	0.57
5	15	Me	H	0.82	0.059
6	16	Br	H	1.27	0.042

CONCLUSIONS

The established use of cephalosporins to release a PYR payload upon β -lactam cleavage is here shown to lead to agents active against *Mtb* in culture under both R and NR conditions. Although the spectrum of the reported compounds is relatively narrow, some of the agents reported were also active against methicillin-sensitive *S. aureus*. Mechanistic studies, which include time-course measurements of PYR release and control agents bearing a nonreleasable pyridine *N*-oxide, suggest that the activity of these compounds arises both from PYR release, although additional β -lactam-dependent mechanisms cannot be excluded at this time. The mechanism of PYR-mediated killing of *Mtb* is as yet unknown. Metal chelation, of copper in particular, and nonspecific electrophilic reactions with bacterial nucleophiles have been proposed in studies in refs 31 and 77 and other bacteria.^{64,65} A recent study, using a close analogue of PYR, found an upregulation of genes associated with metal toxicity in *Mtb* cells exposed for 2 h to the PYR analogue.³¹ Furthermore, the metal chelator EDTA partially reduced the efficacy of free PYR against *C. albicans* but not against *K. pneumoniae*.⁷⁸ No direct mechanistic inference can be drawn from the observations that PYR led to a marked drop in ATP levels in fungi⁶⁶ and bacteria⁷⁹ and that PYR salts induced membrane depolarization in *Neurospora crassa*.⁸⁰

In conclusion, a hybrid molecule of this nature could open a new chemical space in antibiotic development by allowing the use of moieties that would otherwise be dismissed as potentially toxic on their own, provided that the β -lactam targets the warhead to bacterial cells.

EXPERIMENTAL SECTION

Mycobacterial Strains and Culture Methods. Wild-type *Mtb* strain H37Rv (*Mtb*, ATCC 27294) was grown under

standard replicating (R) conditions of Middlebrook 7H9 broth (supplemented with 10% oleic acid, dextrose, and catalase, OADC) (BD Difco, USA), 0.02% tyloxapol (Sigma), 5% CO₂, and 20% O₂. *M. tuberculosis* strains PM638 (Δ *blaC*) and PM669 (PM638 *attB::blaC*) were grown as previously described.⁴² Colony forming units (CFUs) were plated on 7H11 (BD Difco, NJ, USA) agar supplemented with 10% OADC. Minimum inhibitory concentrations (MIC₉₀) were all conducted in tissue culture treated flat bottom 96-well plates (Corning, USA). The MIC₉₀ value was determined as the concentration of compound that inhibited at least 90% of the bacterial growth measure in the DMSO treated wells. Growth was measured by the optical density at 580 nm. Our studies predominantly used the 4-stress model of nonreplication conditions. Other nonreplicating conditions described in our studies include the BCG stationary phase model and the low oxygen recovery model. The BCG stationary phase model involved *Mycobacterium bovis* BCG (IP173) grown for 40 days at 37 °C without agitation in Middlebrook 7H9 broth medium supplemented with 10% OADC and 0.05% Tween 80 (Sigma). Stocks were quantified and stored at -80 °C. On the day of the experiments, BCG stationary phase stock solutions were thawed in PBS without vortexing and the suspension was diluted and dispensed into the plates at a final concentration of 10⁴ to 5 × 10⁴ CFU per well. Compounds were then added. Plates were incubated at 37 °C and ambient oxygen levels. After 7 days, viability was determined using ATP quantification using BacTiter-Glo (Promega) as per the manufacturer's recommendations. Plates were incubated for 10 min in the dark, and luminescence was read using Envision (PerkinElmer). The low oxygen recovery assay (LORA) was conducted as previously described³² in which a *luxAB* (luciferase) containing strain of *Mtb* H37Rv was diluted in Middlebrook 7H12 broth (Middlebrook 7H9 containing Casitone, palmitic acid, BSA, and catalase) and dispensed into 96-well plates containing antimicrobial agents. Microtiter plates were placed under anaerobic conditions consisting of oxygen levels of less than 0.16% and incubated for 10 days at 37 °C. Plates were transferred to replicating growing conditions, and CFU/mL were enumerated after a 28 h recovery period. LORA MIC₉₀'s were determined after incubating the microtiter plates for 7 additional days under replicating condition. 100 μL of cultures was then used to measure luminescence, and MIC₉₀ was determined as the lowest compound concentration inhibiting at least 90% of untreated control growth.

Compounds, Materials, and Synthesis. Compounds for the high throughput screen were obtained from the GSK compound collection (Harlow, UK). Hit analogues 1 and 2 and all other compounds subjected to biological evaluation were synthesized in house and had measured purities of >90% (HPLC; see the [Supporting Information](#) for details and experimental procedures). Resazurin, PYR, and cephalothin were purchased from Sigma-Aldrich (MO, USA) while 10 (2-[(carboxymethyl)sulfanyl]pyridine-1-ium-1-olate) was purchased from Enamine (NJ, USA).

Small Molecule Whole Cell Screening. Whole cell screening was conducted at the GSK Diseases of the Developing World campus in Tres Cantos, Spain. At the outset, 1904 compounds were selected on the basis of structural diversity and compound availability from the GSK β-lactam library. Structural families were clustered using the Sphere Exclusion method, and representative compounds from each family were selected on the basis of availability. The

assays were conducted as described²³ in which the compounds were dissolved in DMSO and dispensed onto 384-well plates. Test conditions followed those described^{21,23} with a few modifications. Briefly, virulent H37Rv *Mtb* cells were exposed to compounds under 4 conditions: replicating (R) and NR in the 4-stress model with the addition of 0.5 mM sodium nitrite or without the addition of sodium nitrite. R cells were diluted to an initial inoculum OD₅₈₀ of 0.01 while NR cells were diluted to an OD₅₈₀ of 0.01 or 0.1. R cells were incubated at 37 °C with 20% O₂ and 5% CO₂ in the presence of β-lactams at 10 or 100 μM (note: the MIC₉₀'s of the clinical isolates shown in [Table S2](#) were carried out in the absence of CO₂). The OD₅₈₀ of the plates of R cells were read after 7 days of compound exposure. NR cells were exposed to compounds at 37 °C for 3 days at 1% O₂ and 5% CO₂ followed by a 1:5 dilution in R medium. The cells were then allowed to recover for 5 days at 37 °C at 20% O₂ and 5% CO₂, and then, the OD₅₈₀ was determined. Compounds active at 100 μM were confirmed with fresh stock in a full (100–0.4 μM) dose response curve. Percent growth inhibition data was analyzed using ActivityBase program, and IC₅₀'s and IC₉₀'s were calculated using nonlinear regression analysis. Initial cytotoxicity assays for establishing the SI were conducted at the GSK facility using library compounds and HepG2 cells in a 384-well plate format. Cytotoxicity assays for the *de novo* synthesized compounds were carried out at WCM using a similar protocol but in a 96-well format and two mammalian cell lines.

Preliminary Cidalty Estimation Using Charcoal Agar Resazurin Assays (CARAs). Minimum inhibitory concentrations (MIC₉₀'s) for compounds progressed for further characterization were performed in a 96-well plate; for NR conditions, this MIC₉₀ value was described as the two plate assay²³ in which, after 7 days of compound exposure under NR conditions, 10 μL of cells from these assay plates was diluted 1:21 into R medium. With dual-active compounds, this NR MIC₉₀ value can reflect the activity of the compound under NR conditions and the R activity of compound carried over in the 10 μL. Therefore, cidalty was estimated using CARA plates. 96-well CARA plates were prepared, and assays were carried out as described.^{7,81} Briefly, 4 g/L of activated charcoal was added to 7H11 agar with glycerol, and 200 μL/well was dispensed into a 96-well plate. After mycobacterial cells were exposed to a dose range of compounds or vehicle control, 10 μL of cells was added to the surface of each well. The level of cidalty was estimated by measuring the level of growth on each well. Growth was assessed after 10 days for R cells and 12–15 days for NR cells. Thus, to each well, we added 50 μL of phosphate buffered saline solution containing 50 μL of 0.02% tyloxapol and 0.01% resazurin in phosphate buffered saline. Plates were developed for 1 h at 37 °C, and the level of fluorescence was measured at an excitation of 530 nm and emission of 590 nm. The CARA_{MBC} was measured as the compound concentration at which the fluorescence drops to <1% of the average DMSO value.

PYR *in Vitro* Detection Assay. A colorimetric ferric nitrate method was used to estimate the concentration of PYR in solution as described⁴⁰ with some modifications. In a 96-well plate, test solution (e.g., bacterial media) and 0.01 M ferric nitrate (dissolved in 0.1 N sulfuric acid) were mixed 1:1 and incubated with shaking for 5–10 min at room temperature. The absorbance of the solution was measured at 600 nm. PYR concentrations were determined using a linear standard curve of sodium PYR.

BlaC Purification and Activity Assay. The pET28 vector containing the truncated form of the *Mtb blaC* gene was kindly shared by Dr. John Blanchard (Albert Einstein College of Medicine) and was expressed and purified in a similar fashion to the described protocol.^{10,71} After BL21 (DE3) *E. coli* cells were induced overnight with 0.5 mM IPTG, cells were lysed in 50 mM Tris (pH 8.0) containing 500 mM NaCl and 30 mM imidazole. The soluble extract was mixed with a Ni-NTA bead slurry (Qiagen) and incubated overnight at 4 °C. Beads were then washed twice with the lysis buffer (10× bed volume), and BlaC was eluted with lysis buffer containing 250 mM imidazole. After dialysis in tris buffered saline (TBS), BlaC was concentrated and gel filtration was performed using Superdex 16/200 (GE) to exclude aggregates. Activity assays were performed in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.4, containing 20 μg/mL of BSA.

Mammalian Cell Toxicity Assay. Compound toxicity of compounds synthesized in house was measured using HepG2, a human liver hepatocellular carcinoma cell line, and Vero cells, a green monkey kidney epithelial cell line. HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, 10 mM HEPES, and 1% nonessential amino acids. Vero cells were grown in DMEM containing 2% FBS, 1 mM pyruvate, 2 mM glutamine, and 10 mM HEPES. Using a TC-treated 96-well plate, 1 × 10⁴ cells/well were seeded and incubated at 37 °C with 5% CO₂ for 24 h before cells were washed with PBS and fresh medium containing compound was added. Cells were then incubated for an additional 2 days in the presence of each of the compounds. HepG2 cell viability was determined using CellTiterGlo (Promega). Vero cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega)/phenazine methosulfate (PMS) (Sigma) colorimetric assay. For the MTS/PMS assay, culture medium was removed and replaced with phenol-free DMEM culture medium. A fresh mixture of MTS and PMS was added and incubated for 2 h, and absorption was determined at 570 nm. An aliquot of the cell medium was used to estimate PYR levels using the PYR detection assay described above.

Antimicrobial Spectrum. *Staphylococcus aureus* MSSA (ATCC25923), *Staphylococcus aureus* MRSA (ATCC43300), *Klebsiella pneumoniae* SHV-1 (ATCC700603), *K. pneumoniae* (ATCC35657), *Enterobacter cloacae* (ATCC13047), and *Pseudomonas aeruginosa* (ATCC27853) were grown in Mueller-Hinton medium (Sigma). *Enterococcus faecium* VSE (ATCC19434) and *E. faecium* VRE (ATCC51559) were grown in brain heart infusion media (Sigma). MIC₉₀ values were determined using cells at an OD₆₀₀ of 0.001 in a 96-well format in which drug was tested in 2-fold dilutions. OD₆₀₀ of the cultures was read after 24 h of compound exposure. The MIC₉₀ value was calculated as the concentration that inhibited at least 90% of growth of the DMSO treated cells. All MIC₉₀ analyses were conducted in triplicate and reproduced in two to three independent experiments.

Plasma Stability. The stability of 73 compounds from the screen was carried out using plasma from female CD-1 mice (Bioreclamation). Stability samples consisted of 5 μL of stock compound solution in 100% DMSO and 95 μL of plasma to a final 10 μg/mL concentration. The samples were incubated at 37 °C with shaking. Ten μL was removed at each time point and combined with 100 μL of 1:1 acetonitrile/methanol, 10 ng/mL of verapamil (Toronto Research Chemicals, Inc.), and

10 μL of acetonitrile/water. Samples were analyzed by LC-MS/MS using the Q-Extractive high-resolution mass spectrometer (Thermo Fisher Scientific). Stability in plasma from other species was similarly carried out. Mouse, human, and nonhuman primate plasma stability for compound 4 was independently verified by Wuxi Biologics (Wuxi, China). Stability was calculated as the percent remaining of the parent compound compared to the initial concentration; otherwise, assays for each species were conducted once.

Microsomal Stability. Liver microsomes from the indicated species (0.5 mg/mL) were suspended in 50 mM phosphate buffer at pH 7.4 containing 4 mM MgCl₂ and 1 mM NADPH. Compounds were added to a final concentration of 0.5 μM, and the percent of parent compound remaining was monitored by LC-MS/MS.

In Vivo Pharmacokinetics in CD-1 Mice. Initial PK data was gathered by administering a single dose of 1a or 8 to two mice per compound via oral gavage at 25 and 20.8 mg/kg, respectively, and plasma levels of parent compound were monitored with LC-MS/MS. Compounds were suspended in 5% *N,N*-dimethylacetamide/60% PEG300/35% dextrose in water. PK studies for compound 8 were repeated using additional mice and with some modifications. For further oral (po) and peritoneal (ip) studies, compound 8 was dissolved in 0.5% carboxymethyl cellulose/0.5% Tween 80. For intravenous administration (iv), the compound was administered in 0.9% saline solution. Mouse bleeds were conducted via tail snips. Mice were active and showed no abnormal behavior during the course of the studies. Bioavailability (*F*) was calculated as

$$F = \frac{\text{AUC}_{\text{extravenous}}/\text{dose}_{\text{extravenous}}}{\text{AUC}_{\text{intravenous}}/\text{dose}_{\text{intravenous}}} \times 100$$

All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfectdis.9b00112](https://doi.org/10.1021/acsinfectdis.9b00112).

Stability of 2a in R and NR media and inoculating effect; activity of 1a and 2a against R and NR media; quantification of PYR release in media; BlaC effect on compound 1a activity; stability of 1a, 1b, 2a, and 8 in microsomes; preliminary pharmacokinetics of 8; activities from high-throughput screen; activity of PYR-containing cephalosporins against drug-sensitive and drug-resistant clinical isolates of *Mtb*; activity of select compounds against bacterial pathogens other than *Mtb*; mammalian cell cytotoxicity of selective agents; synthetic schemes; experimental procedures; characterization of new compounds (PDF)

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Author Contributions

L.L.Q. carried out biological/mechanistic experiments and chose compounds for the study. K.L., S.L.M., Q.N., A.J.P., and C.W.P. selected or designed, synthesized, and characterized compounds for the study. K.A. tested the susceptibility of clinical *Mtb* isolates. K.M., K.S., S.S.K., and P.E.J. characterized compounds against ESKAPE pathogens. L.L.Q., E.P.d.F., M.M.A., S.P.D., and A.M.L. performed screens or provided compounds for screening. M.Z., H.-P.H.L., and V.D. performed and interpreted preliminary pharmacokinetic characterization experiments. L.L.Q., B.G., J.R., and D.Z. carried out and interpreted activity studies vs *Mtb*. S.S., S.L., L.G., and C.R. performed and interpreted LORA and BCG stationary phase assays. L.L.Q., B.G., C.N., and J.A. conceived the project and wrote the paper, and all authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BCG, Bacille Calmette-Guérin; BlaC, class A β -lactamase; CARA, charcoal-agar resazurin assay; MBC, minimum bactericidal concentration; CFU, colony-forming unit; GSK, GlaxoSmithKline; I, inoculum; Ldt, L_{D} -transpeptidase; LORA, low oxygen recovery assay; MIC, minimum inhibitory concentration; *Mtb*, *Mycobacterium tuberculosis*; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium; PMS, phenazine methosulfate; TB, tuberculosis; OADC, oleic acid, dextrose, and catalase; OD, optical density; PBP, penicillin-binding protein; PYR, pyrithione; SI, selectivity index

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