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Novel Cephalosporins Selectively Active on Nonreplicating *Mycobacterium tuberculosis*

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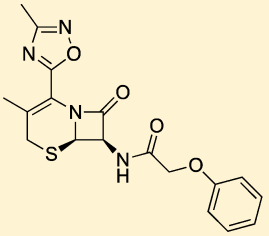
Supporting Information

ABSTRACT: We report two series of novel cephalosporins that are bactericidal to *Mycobacterium tuberculosis* alone of the pathogens tested, which only kill *M. tuberculosis* when its replication is halted by conditions resembling those believed to pertain in the host, and whose bactericidal activity is not dependent upon or enhanced by clavulanate, a β-lactamase inhibitor. The two classes of cephalosporins bear an ester or alternatively an oxadiazole isostere at C-2 of the cephalosporin ring system, a position that is almost exclusively a carboxylic acid in clinically used agents in the class. Representatives of the series kill *M. tuberculosis* within macrophages without toxicity to the macrophages or other mammalian cells.



MIC_{90} for non-replicating *Mtb* 0.88 μg/mL

MIC_{90} for replicating *Mtb* >100 μg/mL

LD_{50} in HepG2 >100 μg/mL

Narrow spectrum

INTRODUCTION

Antibiotics that rapidly kill *Mycobacterium tuberculosis* in axenic culture require months to years to produce the same result in tuberculosis (TB) patients. Accordingly, TB treatment lags far behind that of other bacterial diseases in terms of treatment duration, number of antibiotics required, toxicity to the host, and cure rates. Although new drugs such as oxazolidinones (linezolid, Pfizer),[1] diarylquinolines (bedaquiline, Janssen),[2] and nitroimidazoles (e.g., delamanid)[3] offer hope of shortening TB therapy and reducing mortality in patients whose TB is resistant to the standard regimen, there is an urgent need to discover additional anti-TB drugs.

Even when *M. tuberculosis* is genetically sensitive to existing drugs, a small fraction of a replicating population survives exposure to each such drug in vitro. Such bacteria are termed "persisters". They display class I phenotypic tolerance[4] in that, when the drug is removed and the persisters are allowed to replicate, application of the drug at the same concentration again kills the vast majority. Mechanisms of class I persistence range from temporary nonreplication of a small subpopulation[5] to heterogeneous expression of proteins that activate prodrugs and misincorporation of amino acids into proteins.[6−9] Genetically susceptible *M. tuberculosis* can also display class II phenotypic tolerance when external stresses, such as those imposed by the host immune system, prevent most of the population from replicating.[4,10−12] Ideally, TB should be treated with a combination of drugs such that bacteria displaying class I phenotypic tolerance to any one of them are killed by at least one of the others, and at least one of the drugs can kill nonreplicating *M. tuberculosis* that display class II phenotypic tolerance.

Some of the conditions in the host that can drive *M. tuberculosis* into replication arrest and class II phenotypic tolerance include residence in interferon γ (IFNγ)-activated macrophages that traffic the bacilli to acidified phagosomes and expose them to nitrosative and oxidative stress[13−15] or release of *M. tuberculosis* into the hypoxic milieu of necrotic granulomas.[16] The search for compounds active against nonreplicating *M. tuberculosis* has been pursued in a variety of in vitro nonreplicating models.[17−30] We recently developed a high throughput screening platform to identify small molecules that kill class II persistent *M. tuberculosis* that are rendered nonreplicating by a combination of four host-relevant

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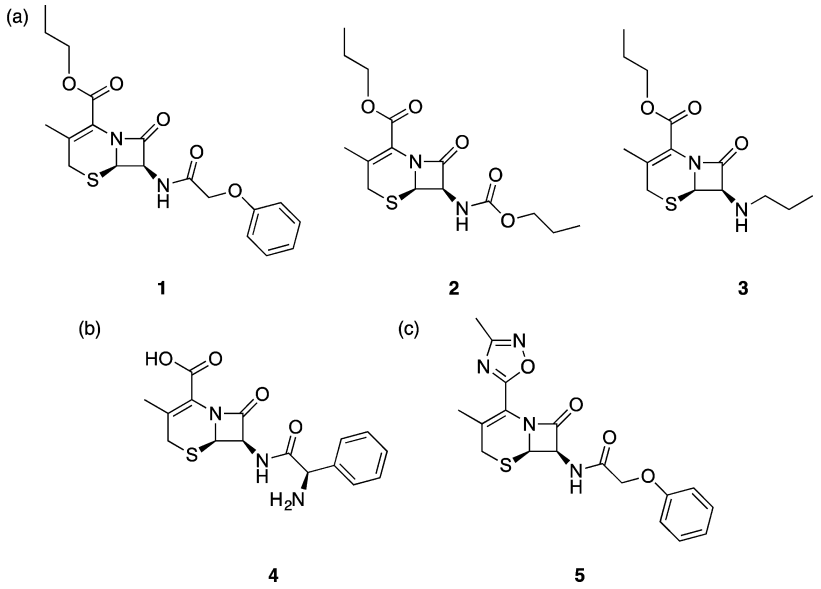

Figure 1. Structures of (a) cephalosporins **1**–**3** selectively active on nonreplicating *M. tuberculosis*, (b) for an inactive analogue, the clinically used antibiotic cephalexin, and (c) the C-2 oxadiazole cephalosporin **5**.

Table 1. Primary Screening Actives that Target Non-Replicating *M. tuberculosis*

compound	NR-MIC_{90} 3 days OD_{580} = 0.1 (μg/mL)	NR-MIC_{90} 6 days OD_{580} = 0.1 (μg/mL)	NR-MIC_{90} 3 days OD_{580} = 0.01 (μg/mL)	NR-MIC_{90} 6 days OD_{580} = 0.01 (μg/mL)	R-MIC_{90} 3 days OD_{580} = 0.01 (μg/mL)	HepG2 LD50 (μg/mL)	d6: % remaining in PBS	d6: % remaining NR medium	d6: % remaining NR medium + 0.5 mM $NaNO_2$
1	1.76	1.05	0.90	0.64	>100	>75.69	80	100	100
2	2.61	0.52	3.11	n.t.[a]	55.44	>100	80	100	100
3	2.69	0.45	0.7	n.t.[a]	>100	>100	100	100	40
cephalexin **4**	61.35	70.42	97.93	73.04	33.99	>100	50	100	100

[a]n.t. = not tested.

conditions:[13−15,21,31−35] low pH (5.0), a flux of nitric oxide (generated from 0.5 mM nitrite at that pH), hypoxia (1% O_2), and low concentrations of a fatty acid (0.05% butyrate) as the carbon source instead of the conventional carbon sources dextrose and glycerol.[21,36] Here, we report that this screening protocol has led to the discovery of the first cephalosporins, to our knowledge, that are selectively active against bacteria in a nonreplicating state. We describe an initial analysis of their structure−activity relationship.

RESULTS

Identification of Cephalosporins Active on Non-replicating *M. tuberculosis*. A high-throughput screening campaign against replicating and nonreplicating *M. tuberculosis* was carried out using a library of compounds from an in-house screening collection assembled at the University of Kansas. This screen led to the identification of three cephalosporin esters[37] (**1**−**3**, Figure 1a) whose activity was unique to nonreplicating *M. tuberculosis*. For comparison, cephalexin **4**, a broad-spectrum antibiotic in clinical use, was also tested in the multistress nonreplicating model but found to be inactive. The three hit molecules were resynthesized to >96% purity and displayed MIC_{90}s against nonreplicating *M. tuberculosis* of 1.8−2.7 μg/mL and 0.5−1.1 μg/mL during exposures lasting 3 or 6 days, respectively (Table 1). Initial results were obtained with a strain of *M. tuberculosis* whose dual auxotrophy for pantothenate and lysine increases its safety for laboratory personnel,[38,39] and key results for select molecules were verified using virulent, wild-type *M. tuberculosis* H37Rv. Given the propensity of β-lactams for inoculum effects,[40] we also determined the MIC_{90}s against nonreplicating *M. tuberculosis* using a 10-fold lower inoculum of A_{580} of 0.01. The results at 3 days (0.7−3.1 μg/mL) were similar to those found for the higher inoculum cultures exposed for 6 days. Activity against replicating *M. tuberculosis* and against human HepG2 hepatoma cells was not seen up to the highest concentration tested (100 μg/mL), and the best selectivity index was ≥250. Thus, the activity of these compounds against *M. tuberculosis* was directly dependent on time of exposure and the state of nonreplication, inversely dependent on concentration of the bacteria, and selective for *M. tuberculosis* over human cells.

Stability in Cell-Free PBS and Nonreplicating Medium. Because some molecules are chemically unstable in the multistress model of nonreplication,[21,35,36] compounds **1** and **2** were tested and found to be stable for up to 6 days in cell-free PBS and nonreplicating medium containing or omitting $NaNO_2$ (Figure 2a and b). However, **3** was unstable in cell-free nonreplicating medium containing $NaNO_2$ (Figure 2c; summarized in Table 1). For comparison, cephalexin was partially unstable in cell-free PBS and stable in cell-free nonreplicating medium either containing or lacking $NaNO_2$ (Figure 2d).

Structure−Activity Relationship (SAR) Studies. These promising results prompted us to undertake an initial structure−activity relationship survey. For each new analogue, we determined the activity against *Mtb* under both non-

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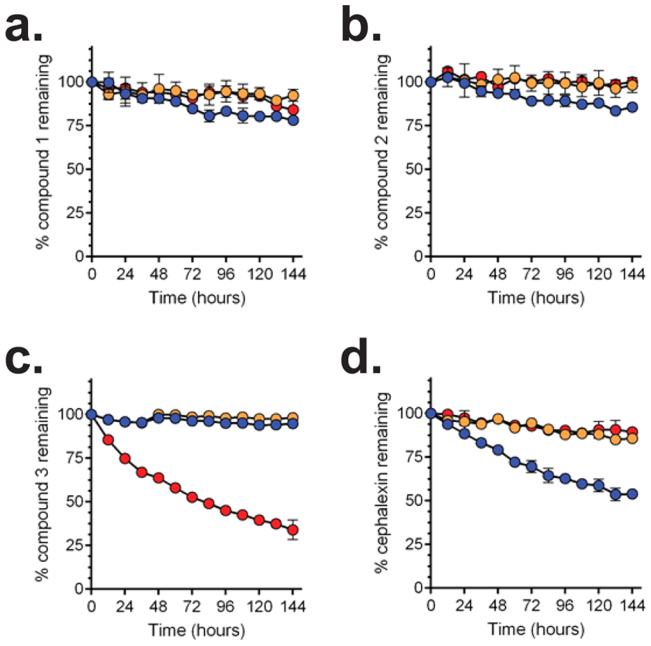

Figure 2. Cell-free stability of primary screening hits. Molecules were incubated at 37 °C in PBS (blue) or nonreplicating medium without (orange) or with (red) $NaNO_2$. Data are averages of replicate samples ± standard deviation.

replicating (NR) and replicating (R) conditions. In addition, each compound was assayed for cytotoxicity against HepG2 cells. For the present discussion, analogues are presented in Tables 2–5 according to the chemical class investigated.

To test if the ester moiety in compounds **1**–**3** was essential for activity, we synthesized five free acid analogues of **1** (**9**) or related molecules and tested three commercially available cephalosporins bearing C-2 carboxylic acids (cephalexin, cefdinir, and cephalothin). The activity of all the cephalosporin carboxylates tested against nonreplicating *M. tuberculosis* ranged from ∼50 to >100 μg/mL (Table 2), signifying the importance of an ester moiety at C-2. To determine if additional functional groups could be tolerated at this position, we prepared and tested analogues containing various amides, alcohols, and ethers at C-2 (Table 2, compounds **5**–**18**). All were found to be inactive. However, replacement of the ester moiety by the isosteric 1,2,4-oxadiazole in analogue **5** resulted in a compound that was close in activity to **1** (Figure 1c and Table 2). Moreover, compound **5** was stable in cell-free nonreplicating medium containing $NaNO_2$ (Figure 3).

To determine if simply adding ester groups or an oxadiazole to C-2 of a classical cephalosporin would confer activity against nonreplicating *M. tuberculosis*, we made cephalexin analogues **4a**–**c** bearing such modifications. Testing determined that analogues **4a** and **4c** were poorly active against nonreplicating *M. tuberculosis* and completely inactive against replicating *M. tuberculosis* (Table 3). *n*-Propyl ester **4b** was slightly more active with an NR-MIC_{90} of 15.5 μg/mL.

Given these results, we chose to concentrate on exploring the effect of C-2 and C-7 amino substitution in two series of analogues: the esters (Table 4) and the oxadiazoles (Table 5). All compounds were synthesized as summarized in Scheme 1. Esters were generated from commercially available 7-amino-deacetoxycephalosporanic acid (7-ADCA) by amidation, followed by esterification. The oxadiazole series was prepared by installing the desired oxadiazole onto the C-2 of carboxylic acid A[41] as shown in Scheme 1 below. Compounds were typically purified by mass-directed HPLC fractionation and rendered in purities of ≥95% for biological evaluation.

Numerous analogues in both series of compounds were active against nonreplicating *M. tuberculosis*. The activity profiles of these molecules were responsive to these basic chemical changes, which suggested that the compounds were exerting their activity through action at a discrete cellular target. For example, a preference for longish and unbranched esters at C-2 was generally observed with ethyl, propyl, and butyl esters being preferred. Activity was sharply diminished for analogues containing propargyl groups at this position (e.g., compounds **4**, **6**, and **22**), although benzyl esters were tolerated (compounds **14**, **24**, and **29**). Recalling that cephalexin analogues **4a** and **4b** containing a side chain bearing a primary amine were poorly active, we concentrated our initial SAR on neutral C-7 amide moieties (although the single C-7 propyl amine examined, compound **3**, did have significant activity). Most of the compounds examined bore a substituted 3-phenylpropamide side chain or its ethereal analogue (e.g., compound **13**). We also observed that moving the double bond from the $\Delta^{2,3}$ to the $\Delta^{3,4}$ had only a modest effect (cf. compounds **34** and **35**).

Among the C-2 oxadiazoles (Table 5), similar trends were observed with a few addenda. Here, a wider range of carbamates, including a single carbothioate, were prepared and found to be active (compounds **14**–**17**). In this series, we noted that although compounds bearing *para* electron-withdrawing substituents retained excellent potency, in many cases measurable levels of cellular toxicity were also observed. Particularly notable examples included some *p*-Cl and 3,4-dichloro analogues (compounds **5**, **12**, **21**, and **29**).

Physicochemical Properties and Metabolic Stability. Compounds **1** and **5** were chosen as representative molecules of the alkyl ester and oxadiazole classes of cephalosporins that are active against nonreplicating *M. tuberculosis,* whereas cephalexin, cefdinir, and cephalothin were chosen as representatives of cephalosporins lacking such activity. The active cephalosporins shared higher values for clogP and pK_a, whereas other properties such as H-bond donors, H-bond acceptors, molecular weight, heavy atom count, and rotatable bonds were similar (Table 6).

We next determined the hydrolytic stability of these compounds under strongly acidic conditions, such as would be encountered in the stomach. Both **1** and **5** were more stable at pH 2 (100% remaining after 4 h) than cephalexin (∼74% remaining) (Table 7), whereas all three compounds were stable at pH 7 and degraded in base (pH 12). Compound **5** and cephalexin were soluble at 84 and 76 μM at pH 7.4, respectively, whereas **1** was less soluble at 23 μM (Table 7).

Parallel artificial membrane permeability assays (PAMPA) predicted that both **1** and **5** would be membrane permeable (Table 8). However, unlike cephalexin, both **1** and **5** were rapidly metabolized by mouse liver microsomes (Table 8). Compounds **1** and **5** were less susceptible to metabolism by human liver microsomes with half-lives of ∼80 min and CL_{int} values suggestive of slow metabolism (Table 8).

Next, we assessed the stability of compounds **1** and **5** in mouse plasma to determine the feasibility of testing these compounds for activity in a mouse model of tuberculosis. Both compounds **1** and **5** were completely transformed in mouse plasma in <5 min (Figure 4a). In human plasma, compounds **1** and **5** had half-lives of approximately 2–3 h (Figure 4b).

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Table 2. Survey of C-2-Substituted Cephalosporins[b]

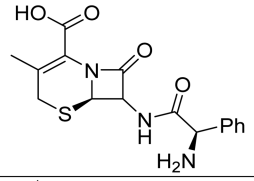
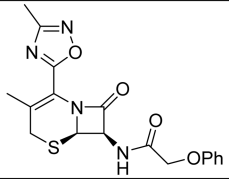
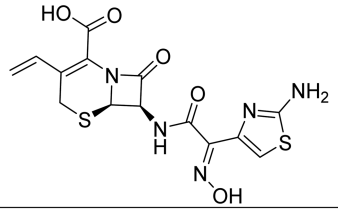
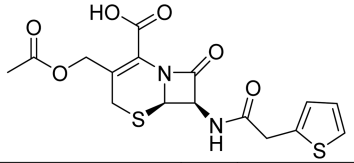
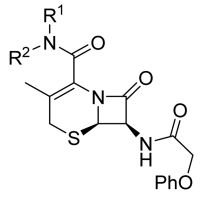
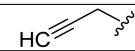
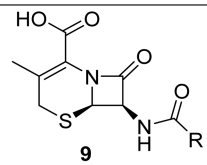
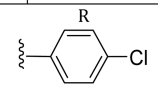
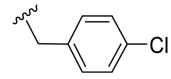
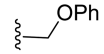
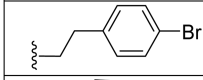
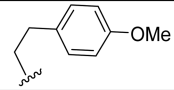
entry	structure	compound	CLog P	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.0 1 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
1		cefalexin, cephalexin **4**	-2.15	100	73.04	33.99	>100.00
2		**5**	1.12	1.85	0.88	>100.00	>100.00
3		cefdinir **6**	-1.67	>100	77.84	4.11	>100.00
4		cephalothin **7**	0.02	>100	95.53	>100.00	>100.00
5		R¹ = H, R² = H	**8a**	-0.18	>100	59.68	>100.00	n.d.
6		R¹ = H, R² = Me	**8b**	0.04	>36.14	>36.14	>100.00	n.d.
7		R¹ = H, R² = 	**8c**	0.27	>38.54	>38.54	>100.00	>100.00
8		R¹ = *n*-Pr, R² = OH	**8d**	0.92	>40.55	>40.55	>100.00	n.d.
9	 **9**		**9a**	1.57	>35.28	>35.28	>100.00	>100.00
10			**9b**	1.55	>36.68	>36.68	81.38	>100.00
11			**9c**	0.63	>34.84	>34.84	>100.00	n.d.
12			**9d**	2.16	>42.53	>42.53	96.13	>100.00
13			**9e**	1.23	>37.64	>37.64	>100.00	n.d.

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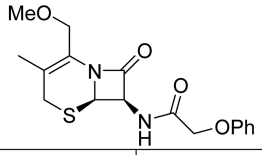
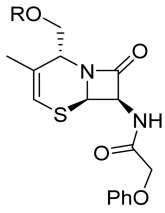
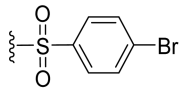
Table 2. continued

entry		R	compound		NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.0 1 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
14			**10**	0.80	>100.00 [a]	n.t.	>96.74	n.d.
15		H	**11a**	0.21	>100.00 [a]	n.t.	>100.00	>100.00
16		Me	**11b**	0.86	>34.84	>34.84	>87.82	n.d.
17		*n*-Pr	**11c**	1.74	>100.00 [a]	n.t.	>100.00	n.d.
18			**11d**	3.03	>55.34	>55.34	>83.24	n.d.

[a]These data were from a 3-day exposure to compound. [b]n.d. = not determined. n.t. = not tested.

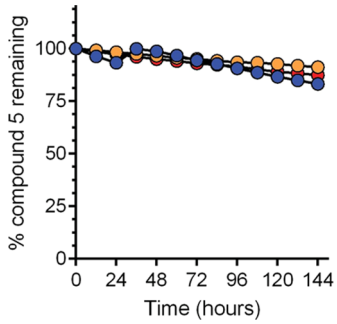


Figure 3. Cell-free stability of **5**. Compound **5** was incubated at 37 °C in PBS (blue) or nonreplicating medium without (orange) or with (red) $NaNO_2$. Data are averages of replicate samples ± standard deviation.

Cephalexin **4** was stable in plasma from both species (Figure 4a and b).

Selective Bactericidal Action of Cephalosporins on Nonreplicating *M. tuberculosis*. Narrow spectrum bactericidal activity is preferred for TB drugs for two reasons. First, TB treatment is protracted, and long-term exposure to broad-spectrum antibacterial agents can precipitate severe and sometimes fatal intestinal dysfunction, such as that caused by overgrowth of *Clostridium difficile*. Second, efficacy of a given drug against other bacterial infections can prompt its use in the community, including in people who have undiagnosed TB. Monotherapy of TB often selects for emergence of genetically resistant strains. The spread of such strains in the community would render the new drug progressively less useful for the treatment of TB. Hence, it was important to test the antimicrobial spectrum of the new cephalosporins against other bacteria. Compounds **1** and **5** had MIC_{90}s > 100 μg/mL against replicating *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Mycobacterium bovis* BCG as well as against the fungus *Candida albicans* (Figure S1).

To determine the extent of bacterial kill, we exposed nonreplicating wild-type *M. tuberculosis* at an OD_{580} of 0.01 (low inoculum) to **1** and **5** for 7 days (Figure 5). At ∼ 0.7−0.8 μg/mL, both compounds reduced colony-forming units (CFUs) by 2 log_{10}. Compounds **1** and **5**, at 3 and 10 μg/mL, respectively, reduced CFU to the extent that there were no recoverable colonies when 10 μL of undiluted sample was plated (≥3.4 log_{10} kill). Thus, no class I phenotypic tolerance was observed. Bactericidal activity of **1** was not enhanced by the addition of a β-lactamase inhibitor, clavulanate, and addition of clavulanate did not lead to activity of **3**, **2**, or **1** against replicating *M. tuberculosis* (data not shown). In contrast, clavulanate enhanced the replicating MIC_{90} of Meropenem 4-fold.

Representative cephalosporins (compounds **5**, **12e**, **12i**, **18d**, **19d**, **21b**, **22c**, and **23**) from the preliminary SAR campaign (Tables 2−5) were tested for activity against nonreplicating wild-type *M. tuberculosis*. Analogues derived from compounds **1** and **5** were bactericidal in this assay (Figure S2).

Reactive Nitrogen Species Enhance Bactericidal Activity of 1 and 5 against Nonreplicating *M. tuberculosis*. The activity of **1** against nonreplicating *M. tuberculosis* increased in relation to the concentration of $NaNO_2$ (Figure 6a), whereas that of rifampicin did not at ≤0.5 mM $NaNO_2$ (Figure 6b). At 1 mM $NaNO_2$, double the concentration used in the nonreplicating screening, we observed nitrite-dependent killing with rifampicin as well. We then tested both **1** and **5** for nitrite-dependence by coupling the outgrowth to a CFU-surrogate assay (charcoal agar resazurin assay; CARA)[42] that determines the approximate concentration of compound leading to ≥2−3 log_{10} CFU reduction as reflected by the ability of survivors to convert resazurin to a fluorescent product. Both **1** and **5** decreased fluorescence in a dose-dependent manner that was strongly enhanced by the addition of $NaNO_2$ (Figure 6c and d). As observed for many of the β-lactams in this study, the activities of both **1** and **5** were more potent at a 10-fold lower inoculum of 0.01 and 7-day exposure (Figure 6d). Both compounds displayed nitrite-independent activity at the lower inoculum (Figure 6c and Figure 6d). Thus, nitrite contributed to a 32- to 64-fold enhancement of **1**'s activity, but activity was not strictly dependent on an exogenous source of nitrite (Figure 6d).

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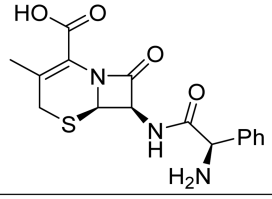
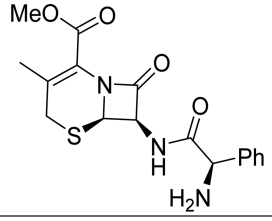
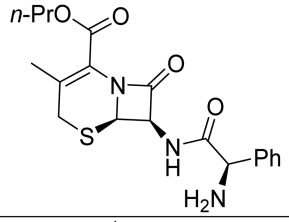
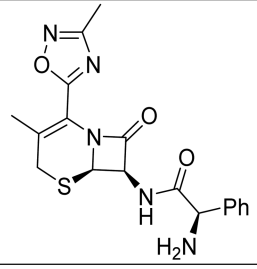
Table 3. C-2 Ester and Oxadiazole Analogues of Cephalexin

entry	structure	compound	CLogP	NR d7 OD=0.1 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
1		cefalexin, cephalexin **4**	-2.15	100	33.99	>100.00
2		**4a**	0.50	61.78	>100.00	>100.00
3		**4b**	1.38	15.46	>100.00	>100.00
4		**4c**	0.63	44.05	>100.00	>100.00

Nonreplicating-Active Cephalosporins Kill *M. tuberculosis* in Macrophages. Wild-type *M. tuberculosis* is typically growth-arrested, or replicates slowly, in activated macrophages, due in part to phagosomal acidification and macrophage production of reactive nitrogen species (RNS).[13,14] The multistress nonreplicating assay conditions were designed in part to mimic this phagosomal microenvironment.[21,35] We hypothesized that cephalosporins active in the nonreplicating model might be bactericidal against intracellular *M. tuberculosis*. To test this, we stimulated mouse bone marrow-derived macrophages with IFNγ, or left them unstimulated, infected them with wild-type *M. tuberculosis*, and treated them with **1**, **5**, or diluent alone. We observed approximately 1−2 $\log_{10}$ CFU reduction of intracellular *M. tuberculosis* in activated macrophages treated with **1** or **5** with no apparent toxicity to the macrophages. Compound **5**'s bactericidal activity against intracellular *M. tuberculosis* was strictly IFNγ dependent (Figure 7a and b).

DISCUSSION

To our knowledge, this is the first report of β-lactams that only kill a given bacterium when it is nonreplicating, and the first report of β-lactams with activity against any one bacterial species that lacks broad-spectrum antibacterial activity. Early studies by Tuomanen et al. demonstrated that although many β-lactams lack activity against nongrowing cells, a minority killed starved, nonreplicating *Escherichia coli* and *Streptococcus pneumonia*.[43,44] Similar findings were recently observed in *M. tuberculosis*, as the combination of Meropenem and the β-lactamase inhibitor clavulanate killed both replicating and hypoxic, nonreplicating *M. tuberculosis*.[45] Meropenem-clavulanate lacked activity against nonreplicating *M. tuberculosis* in the conditions studied here. In addition to hypoxia, our conditions included a low pH, a flux of reactive nitrogen species, and a fatty acid carbon source. Faropenem was also reported to kill both replicating and nongrowing *M. tuberculosis*.[46] Like Meropenem, faropenem was inactive in our multistress model of nonreplication. The novel cephalosporins described here did not acquire activity against replicating *M. tuberculosis* when we included clavulanate in the assays.

Structurally, the two main classes of compounds explored herein differ from clinically used cephalosporins by the lack of a carboxylic acid moiety at C-2 (a notable exception being the prodrug cefuroxime axetil), and indeed, we showed that carboxylic acid **9** is inactive against nonreplicating Mtb. Early in this project, we considered whether the screening hit propyl ester was functioning as a prodrug, but the successful

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Table 4. SAR of C-2 Cephalosporin Esters[b]

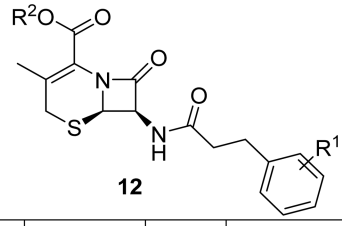
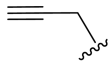
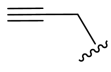
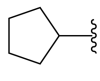
entry	R^1	R^2	compound	CLogP	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.01 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
			 **12**					
1	H	Me	**12a**	1.77	1.27	1.04	>100.00	>100.00
2	H	Et	**12b**	2.12	1.15	0.47	>100.00	>100.00
3	H	*n*-Pr	**12c**	2.65	1.24	0.6	53.64	>100.00
4	H		**12d**	1.99	19.97[a]	1.11[a]	>100.00	>100.00
5	H	*n*-Bu	**12e**	3.09	<0.24	0.21	>100.00	>100.00
6	*p*-Cl		**12f**	2.60	7.85[a]	0.56[a]	>100.00	>100.00
7	*p*-Cl	*n*-Bu	**12g**	3.69	1.24	0.72	>100.00	>100.00
8	*p*-OMe	Me	**12h**	1.61	3.28	0.39[a]	59.82	>100.00
9	*p*-OMe	Et	**12i**	1.97	0.51	0.4	21.37	>100.00
10	*p*-OMe	*n*-Pr	**12j**	2.49	<0.20	<0.20	>100.00	>100.00
11	*p*-OMe		**12k**	2.91	2.95	1.57	>100.00	n.d.
12	*p*-OMe	*n*-Bu	**12l**	2.93	0.26	0.24	>100.00	>100.00
13	*p*-OMe		**12n**	2.96	0.96	0.84	44.33	n.d.
14	*p*-OMe	Benzyl	**12o**	3.33	0.42	0.39	>100.00	>100.00

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Table 4. continued

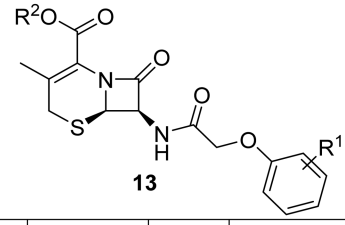
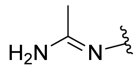
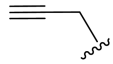
entry	R^1	R^2	compound	CLogP	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.01 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
		 **13**						
15	H	Me	**13a**	1.00	2.93	2.07	>100.00	>100.00
16	H	Et	**13b**	1.36	1.71	0.66	57.85	>100.00
17	H	*n*-Pr	**1**	1.88	1.05	0.64	>100.00	>100.00
18	H		**13c**	0.26	8.12	5.60	>100.00	n.d.
19	*p*-Cl	Me	**13d**	1.61	1.25	0.95	>100.00	>100.00
20	*p*-Cl	Et	**13e**	1.97	2.38	1.07	>100.00	41.02
21	*p*-Cl	*n*-Pr	**13f**	2.49	1.34	0.66	>100.00	49.22
22	*p*-Cl		**13g**	1.84	3.95	1.53	>100.00	63.41
23	*p*-Cl	*n*-Bu	**13h**	2.93	1.01	0.43	>100.00	>100.00
24	*p*-Cl	Benzyl	**13i**	3.33	8.07	0.87	>100.00	>100.00
25	*p*-OMe	Me	**13j**	0.85	4.28	2.01	>100.00	>100.00
26	*p*-OMe	Et	**13k**	1.20	0.89	0.53	47.10	>100.00
27	*p*-OMe	*n*-Pr	**13l**	1.73	0.81	0.39	>100.00	>100.00
28	*p*-OMe	*n*-Bu	**13m**	2.17	1.12	0.71	>100.00	>100.00

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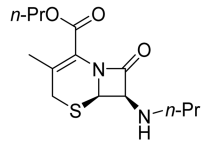
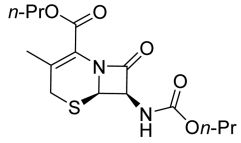
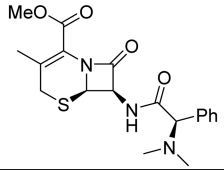
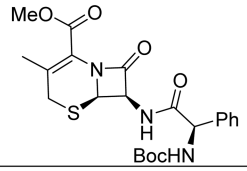
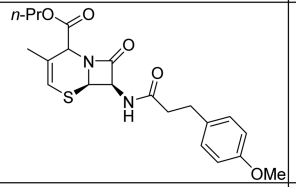
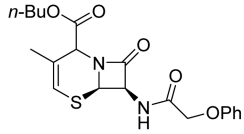
Table 4. continued

entry	R^1	R^2	compound	CLogP	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.01 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
29	*p*-OMe	Benzyl	**13n**	2.57	2.53	1.54	>100.00	>100.00
30			**3**	1.86	2.69[a]	0.70[a]	>100.00	>100.00
31			**2**	1.87	2.61[a]	3.11[a]	55.44	>100.00
32			**14a**	1.32	>38.95	>38.95	>100.00	>100.00
33			**14b**	1.99	18.07	16.51	>100.00	n.d.
34			**15a**	2.16	<0.23	0.21	49.50	>100.00
35			**15b**	2.00	1.28	0.79	>100.00	61.77

[a]These data were from a 3-day exposure to compound. [b]n.d. = not determined.

replacement of this moiety with the corresponding oxadiazole renders this possibility unlikely. A hydrophobic moiety at this position is tolerated, as seen with the *n*-propyl ester and oxadiazole, and possibly preferred, given the fact that the C-2 hydroxymethyl analogue **10** is inactive. Polarity at this position also plays a role given the inactivity of the amide analogous to the active esters. In the preliminary SAR pursued to date, we have also ascertained that biological activity is affected by the amide moiety attached to the central cephalosporin nucleus with chains ending in electron-poor aromatic rings being preferred and a moderate dependence of activity on the length of the chain leading to this point. The role of the β-lactam itself is currently ambiguous; although we know that hydrolytic cleavage of this ring results in an inactive compound, it is not clear whether this is because the β-lactam is essential per se, i.e., in analogy to the generally accepted mechanism of most β-lactams, which involve covalent binding of this group to the target protein, or because of a structural alignment resulting from the cephalosporin ring system.

A question of pressing interest is the molecular target that renders these cephalosporins profoundly active against *M. tuberculosis* in a nonreplicating state. Experiments to address this are underway by a variety of approaches but have not yet yielded an answer, although we note that our observation of structure−activity relationships is consistent with action through one or more specific targets. The canonical role of β-lactams in killing replicating bacteria has been widely accepted as the arrest of peptidoglycan biosynthesis. Disruption of the balance between new peptidoglycan synthesis and peptidoglycan cleavage by hydrolases leads to cellular lysis[47,48] due to a futile cycle in the synthetic pathway.[49] The sensitivity of hypoxic *M. tuberculosis* to Meropenem and clavulanate, albeit significantly less than the sensitivity of replicating *M. tuberculosis*, suggests that *M. tuberculosis* may require peptidoglycan

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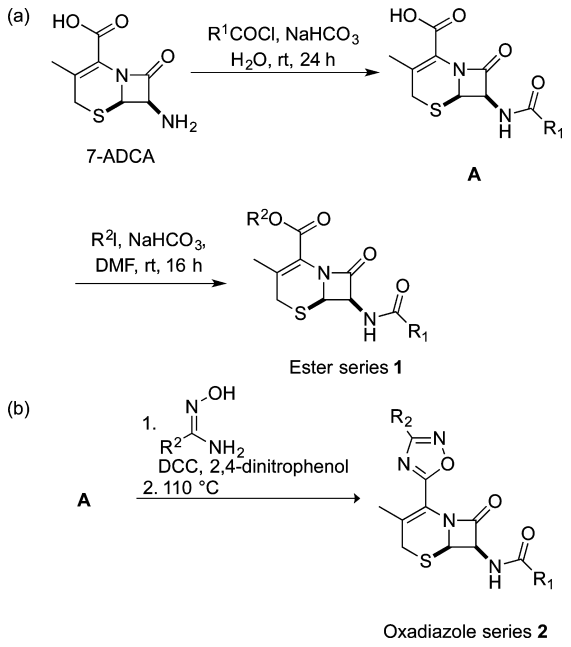
Scheme 1. Synthetic Route for the Preparation of Ester and Oxadiazole Analogues



biosynthesis to survive the hypoxic state.[45] In addition to classical D,D-transpeptidases that catalyze the formation of 4′ → 3′ peptidoglycan cross-links, *M. tuberculosis* may use at least five L,D-transpeptidases (Ldt_{MT1}–Ldt_{MT5}; LDTs) for peptidoglycan 3′ → 3′ cross-linking. The 3′ → 3′ cross-links account for ∼80% of peptidoglycan extracted from *M. tuberculosis* in stationary phase, a form of nonreplication.[50] One of the nonclassical L,D-transpeptidases, Ldt_{MT2}, plays a role in *M. tuberculosis* virulence in a mouse model of infection.[51] Meropenem and other carbapenems bind recombinant $Ldt_{MT1,2,4,5}$.[52] However, there are additional covalent targets of β-lactams, such as signal peptidases and proteases.[53−55] Thus, we anticipate that β-lactams that specifically target nonreplicating populations may have either single or multiple canonical or noncanonical targets.

Two of the cephalosporins with activity against nonreplicating *M. tuberculosis* were stable in PBS and in nonreplicating medium, whether or not the medium contained $NaNO_2$. An equipotent compound, **3**, was unstable. Thus, **3** may have entered the bacilli or otherwise exerted its bactericidal effect before its structure was transformed in the extracellular medium.

In animal and human tuberculosis, *M. tuberculosis* often resides in macrophages,[56] and the ability to kill intracellular bacilli is an important feature of antimycobacterial compounds. Two of the nonreplicating active cephalosporins, compounds **1** and **5**, killed intracellular *M. tuberculosis* but with differential dependences on immune activation. Immune activation of *M. tuberculosis*-infected macrophages leads to profound changes of the phagosomal microenvironment that are anticipated to lead to growth arrest. These changes include phagosomal acidification to approximately pH 4.5 and induction of iNOS, which produces nitric oxide.[14,15] *M. tuberculosis* exhibits variable behavior in mouse bone marrow-derived macrophages, ranging from subexponential replication in nonactivated macrophages to slower replication, no net change in CFU, or a modest decline in CFU in activated macrophages. An inhibitor of dihydrolipoamide acyltransferase (DlaT) selectively kills *M. tuberculosis* and *M. bovis* BCG in vitro when they are nonreplicating, and this compound is effective against *M. bovis* BCG in activated macrophages.[17] Likewise, compound **5** killed intracellular *M. tuberculosis* when the macrophages were immune stimulated, consistent with our hypothesis that compound **5** exerts activity in an acidic, nitrosative phagosome. However, compound **1** killed intracellular *M. tuberculosis* both in the absence and presence of IFNγ activation and thus may possess some activity against replicating *M. tuberculosis* at the concentrations tested.

SUMMARY

The potential ability of β-lactams to treat TB has been suggested for many years but has only recently gained substantial notice with the report of promising results using Meropenem in human trials.[45,46] It would be of considerable interest to test the role of β-lactams that target nonreplicating *M. tuberculosis* in TB therapy in combination with agents active against replicating *M. tuberculosis*. Cephalosporins with activity against nonreplicating *M. tuberculosis* identified in this study, **5** and **1**, were nontoxic, stable in cell-free medium, stable at pH 2 and 7, soluble at pH 7.4, predicted to be membrane-permeable, active in macrophages, and inactive against the other bacterial and yeast species tested. Compounds **1** and **5** were relatively stable when incubated with human liver microsomes. Although compounds **1** and **5** were highly labile in mouse plasma, they were more stable in human plasma with half-lives of 2−3 h. Some analogues of compound **5** were active in the ng/mL range. We are continuing to study the SAR of **5** while seeking its targets.

EXPERIMENTAL SECTION

General Procedure for Synthesis of Esters: Propyl (6*R*,7*R*)-3-Methyl-8-oxo-7-(2-phenoxyacetamido)-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate (1).[37] To (6*R*,7*R*)-3-methyl-8-oxo-7-(2-phenoxyacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (197.0 mg, 0.57 mmol) was added sodium bicarbonate (48 mg, 0.57 mmol) and a minimal amount of water (6.0 mL) to dissolve the starting material. The mixture was stirred at rt for 30 min until all solids were dissolved and then frozen and lyophilized. DMF (10.0 mL) was slowly added followed by 1-iodopropane (0.55 mL, 5.7 mmol). The reaction was stirred at rt for 16 h, then quenched with water, and extracted twice with Et_2O. The Et_2O layers were combined and then washed 3 times with water and once with brine solution. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The residue was purified via MPLC (silica, 30% EtOAc/hexanes → 70% EtOAc/hexanes) to afford the title compound (130.0 mg, 59% yield). $[\alpha]_D^{25}$ +71.2 (*c* 1.45, CH_2Cl_2); IR (film) ν_{max} 1780, 1721, 1687, 1524, 1494, 1228, 755 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.34 (m, 2H), 7.30 (s, 1H), 7.05 (tt, J = 7.7, 1.0 Hz, 1H), 6.94 (m, 2H), 5.88 (dd, J = 9.2, 4.7 Hz, 1H), 5.04 (d, J = 4.8 Hz, 1H), 4.58 (s, 2H), 4.24 (m, 2H), 3.53 (dd, J = 18.3, 1.0 Hz, 1H), 3.22 (d, J = 18.3 Hz, 1H), 2.15 (s, 3H), 1.75 (h, J = 7.4 Hz, 2H), 0.99 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.80, 164.03, 162.35, 157.03, 130.88, 129.96, 129.80, 123.01, 122.52, 115.05, 114.90, 67.51, 67.25, 58.44, 56.94, 30.27, 22.03, 20.18, 10.61; HRMS (ESI-TOF) calcd for $C_{19}H_{22}N_2O_5SNH_4^+$ $[M + NH_4]^+$ 408.1588, found 408.1605.

General Procedure for Synthesis of Oxadiazoles: *N*-((6*R*,7*R*)-3-Methyl-2-(3-methyl-1,2,4-oxadiazol-5-yl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-yl)-2-phenoxyacetamide (5). To a solution of 2,4-dinitrophenol (1.03 g, 5.61 mmol) in CH_2Cl_2 (10 mL) was sequentially added (6*R*,7*R*)-3-methyl-8-oxo-7-(2-phenoxyacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (1.92 g, 5.5 mmol) in a minimal amount of 1,4-dioxane (8 mL) and DCC (1.15 g, 5.6 mmol) in 8 mL of CH_2Cl_2. The mixture was stirred at rt for 30 min, after which the mixture was filtered through a plug of cotton to

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Table 5. SAR of C-2 Cephalosporin Oxadiazoles[b]

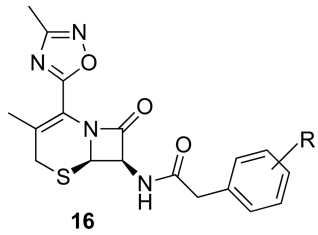
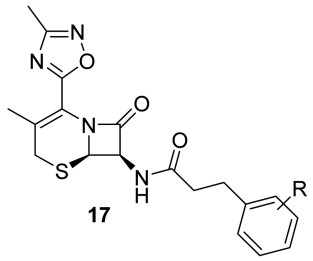
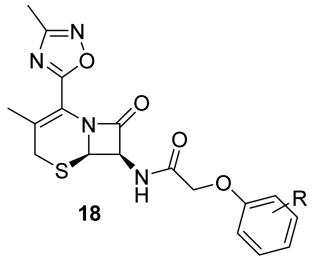
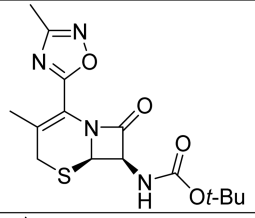
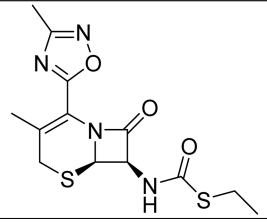
entry	structure		compound	CLog P	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.01 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
1	 **16**	R H	**16a**	1.44	1.61	n.t.	>100.00	>100.00
2		*p*-Me	**16b**	1.94	1.75	n.t.	>100.00	>100.00
3		*p*-OMe	**16c**	1.25	3.25	n.t.	>100.00	>100.00
4		*p*-Cl	**16d**	2.01	1.13	n.t.	>100.00	>100.00
5		3,4-Cl_2	**16e**	2.57	0.28	n.t.	>100.00	18.86
6	 **17**	*p*-OMe	**17a**	1.67	1.68	1.13	>100.00	>100.00
7		*p*-O*n*-Bu	**17b**	2.93	59.36[a]	n.t.	>100.00	74.38
8	 **18**	H	**5**	1.12	1.85	0.88	>100.00	>100.00
9		*p*-Me	**18a**	1.62	7.42[a]	n.t.	>100.00	n.d.
10		*p*-OMe	**18b**	0.93	14.57[a]	n.t.	>100.00	n.d.
11		*m*-Cl	**18c**	1.69	2.59[a]	n.t.	>100.00	n.d.
12		*p*-Cl	**18d**	1.69	1.72	1.2	>100.00	19.51
13		*p*-CF_3	**18e**	2.00	5.26[a]	n.t.	>100.00	n.d.
14			**19a**	1.16	9.16	5.3	>100.00	>100.00
15			**19b**	1.19	1.84	n.t.	>100.00	>100.00

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Table 5. continued

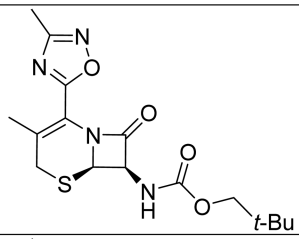
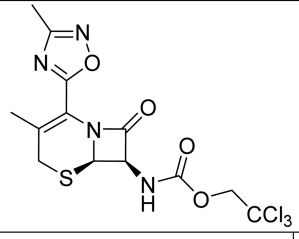
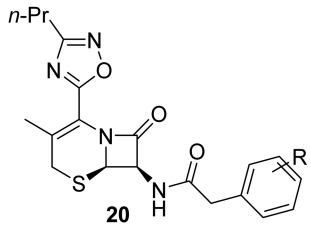
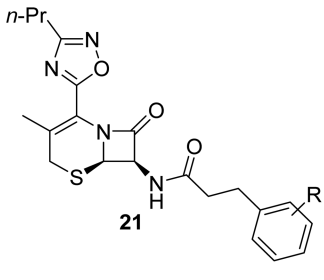
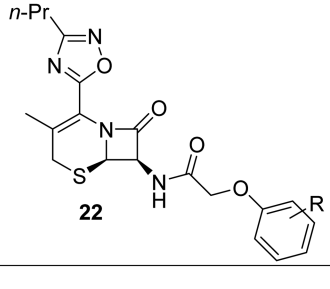
entry	structure		compound	CLog P	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.0 1 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
16			**19c**	1.90	1.03	n.t.	>100.00	>100.00
17			**19d**	1.83	0.92	0.46	>100.00	>100.00
18	 **20**	R H	**20a**	2.51	1.04	n.t.	>100.00	>100.00
19		*p*-Me	**20b**	3.01	0.64	n.t.	>100.00	>100.00
20		*p*-OMe	**20c**	2.31	0.77	n.t.	>100.00	72.38
21		*p*-Cl	**20d**	3.07	2.63[a]	n.t.	>100.00	20.59
22	 **21**	*p*-Me	**21a**	3.43	2.32	0.98	>100.00	>100.00
23		*p*-OMe	**21b**	2.73	0.67	0.37	>100.00	>100.00
24		*p*-O*n*-Bu	**21c**	4.00	0.76	0.47	>100.00	>100.00
25		*p*-Cl	**21d**	3.50	n.t.	n.t.	>100.00	>100.00
26		*o*-Cl	**21e**	3.50	1.27	0.52	>100.00	>100.00
27		*p*-F	**21f**	3.08	0.91	0.48	>100.00	>100.00
28	 **22**	*p*-Me	**22a**	2.69	14.31[a]	n.t.	>100.00	n.d.
29		*p*-Cl	**22c**	2.76	3.87	1.36	>100.00	64.29

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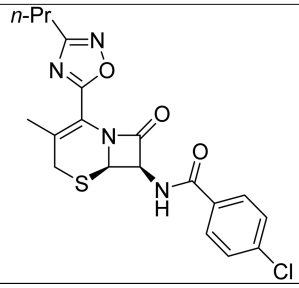
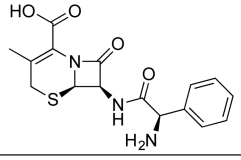
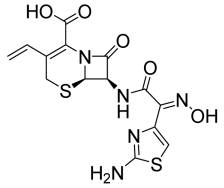
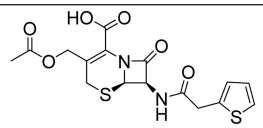
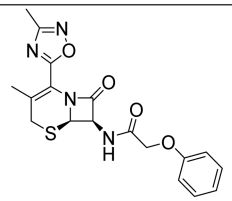
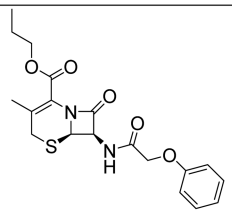
Table 5. continued

entry	structure	compound	CLog P	NR d7 OD=0.1 MIC_{90} (μg/mL)	NR d7 OD=0.0 1 MIC_{90} (μg/mL)	R MIC_{90} (μg/mL)	HepG2 LD_{50} (μg/mL)
30		**23**	3.00	2.28	1.06	>100.00	>100.00

[a]These data were from a 3-day exposure to compound. [b]n.d. = not determined. n.t. = not tested.

Table 6. Predicted Properties of Representative C-2 Ester and Oxadiazole Cephalosporins[a]

Compound	Structure	MW (g/mol)	CLog P	HBD	HBA	pKa	Heavy atom count	PSA (Å^2)	Rotatable bonds
cephalexin **4**		347	-2.20	3	5	3.45	24	113	4
Cefdinir **6**		395	-1.70	4	8	1.74	26	158	5
Cephalothin **7**		396	0.02	2	5	3.63	26	113	7
5		386	1.12	1	5	11.3	27	97.6	5
1		390	1.88	1	4	11.8	27	84.9	8

[a]MW, molecular weight; HDB, H-bond donor; HBA, H-bond acceptor; PSA, polar surface area.

remove the urea. To the filtrate was then added ethylamidoxime (411.0 mg, 5.6 mmol) in CH_2Cl_2 (7 mL), and the mixture was stirred at rt for 4 h. The mixture was then washed twice with sat. aq $NaHCO_3$, filtered, and concentrated. The residue was then placed in a vacuum oven at 110 °C for 16 h, and the resulting residue was purified via MPLC (silica, 100% hexanes → 60% EtOAc/hexanes) to afford the title compound as an orange solid (703.2 mg, 52% yield). $[\alpha]_D^{24}$ +79.6 (*c* 0.72, CH_2Cl_2); IR (film) ν_{max} 1775, 1493, 1331, 1216, 754, 732, 690 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 3H), 7.06 (tt, *J* = 7.4, 1.0 Hz, 1H), 6.96 (m, 2H), 5.95 (dd, *J* = 9.1, 4.8 Hz, 1H), 5.16 (d, *J* = 4.7 Hz, 1H), 4.60 (s, 2H), 3.61 (d, *J* = 18.4 Hz, 1H), 3.35 (d, *J* = 18.3 Hz, 1H), 2.49 (s, 3H), 2.25 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ

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Table 7. Stability and Solubility of 4, 1, and 5

	cephalexin 4	1	5
pH 2 stability (%)[a]	73.7	110	102
pH 7 stability (%)[a]	102	133	101
pH 12 stability (%)[a]	0.000	0.000	0.000
solubility (μM/pH 7.4)[b]	75.8	22.7	83.7

[a]Percent remaining after a 4 h incubation at 37 °C. [b]Determined after shaking at room temperature for 4 h.

Table 8. Preliminary Pharmacokinetic Properties of 4, 1, and 5

	cephalexin 4	1	5
PAMPA (mean Pe(10^{-6}) cm/s)	<0.0001[a]	7.28	13.4
PAMPA (log Pe)	<−9.99	−5.15	−4.87
mouse liver microsomes ($t_{1/2}$, minutes)[b]	stable[c]	<5	<5
mouse liver microsomes, CL_{int} (μL/min/mg protein)[b]	stable	unable to calculate[b]	unable to calculate[b]
human liver microsomes ($t_{1/2}$, minutes)	stable	86.1	76.3
human liver microsomes, CL_{int} (μL/min/mg protein)	stable	8.07	9.13

[a]No compound detected. [b]Both compounds 1 and 5 unstable in assay conditions. [c]No metabolism observed at ≤60 min.

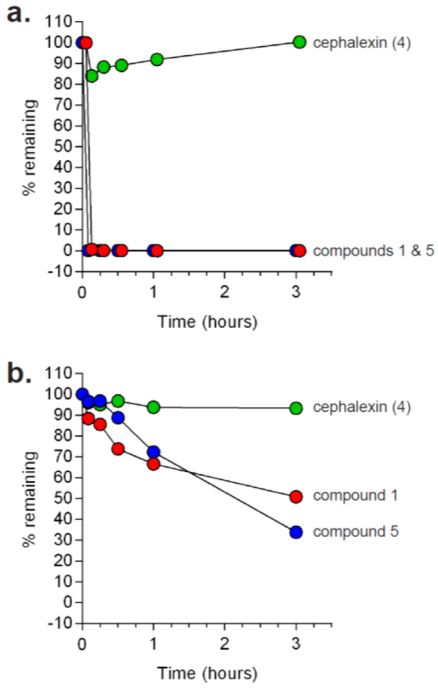

Figure 4. Stability of compounds 1 and 5 in plasma. Compounds 1, 5, and cephalexin (4) were tested for stability in mouse (a) and human (b) plasma at the indicated time points. Stability was inferred by monitoring the parent ion. One of two similar experiments. Compound 1 was tested once in human plasma.

168.79, 167.63, 164.56, 156.99, 130.93, 129.98, 122.56, 117.45, 114.90, 67.25, 58.73, 57.25, 30.02, 20.40, 11.92. HRMS (ESI-TOF) calcd for $C_{18}H_{19}N_4O_4S^+$ $[M + H]^+$ 387.1122, found 387.1088.

Strains and Growth Conditions. Mycobacterial strains and media were prepared as described.[21,35] Briefly, wild-type *M. tuberculosis* H37Rv was cultivated at 20% O_2 and 5% CO_2 in Middlebrook 7H9 bacteriologic medium containing 0.2% glycerol, tyloxapol (0.02%), and 10% OADC supplement, and the *M. tuberculosis* strain mc^{2}6220 (Δ*panCD*Δ*lysA*)[38,57] was grown in similar medium with minor

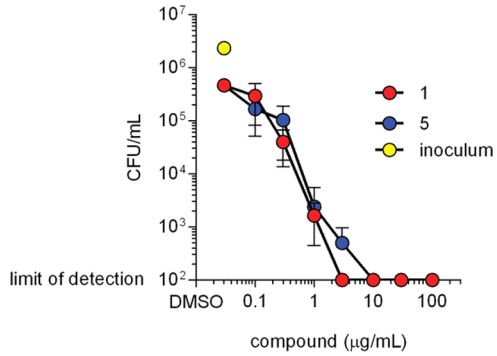

Figure 5. Bactericidal activity of compounds 1 and 5 for nonreplicating *M. tuberculosis*. Nonreplicating wild-type *M. tuberculosis* at an OD_{580} of 0.01 was exposed to compounds for 7 days, and surviving bacilli were enumerated on 7H11-OADC agar plates. The inoculum is shown in yellow. The limit of detection was 1 colony arising from 10 μL of undiluted sample. Error bars represent standard deviations of triplicates. One of two similar experiments.

modifications: additional glycerol (final: 0.5%), OADC supplement, casamino acids (0.05%), L-lysine (240 μg/mL), and pantothenate (24 μg/mL). Cells were rendered nonreplicating at 1% O_2 and 5% CO_2 in a Sauton's-based medium (per liter: 0.5 g of KH_2P0_4, 0.5 g of $MgS0_4$, 0.05 g of ferric ammonium citrate, BSA (0.5%), NaCl (0.085%), tyloxapol (0.02%), L-lysine (240 μg/mL), pantothenate (24 μg/mL), butyrate (0.05%), and 0.5 mM $NaNO_2$).

High Throughput Screen. Molecules from the University of Kansas in-house library were screened using a reported protocol[21,35] with minor modifications. For the replicating screen, 500 nL test agent was added to 50 μL of replicating *M. tuberculosis* mc^{2}6220 at an OD_{580} = 0.01, giving a final concentration of 20 μg/mL and 1% DMSO. After 7 days of incubation at 20% O_2 and 5% CO_2, the OD_{580} was determined. For the nonreplicating screen, *M. tuberculosis* mc^{2}6220 was washed 2× in PBS containing tyloxapol (0.02%; PBS-Tyl) and resuspended in nonreplicating medium containing 0.5 mM $NaNO_2$, and 15 μL of cells were dispensed into 384-well tissue culture plates (Greiner, reference 781091). Cells were exposed to 150 nL of test compounds in DMSO, and plates were incubated for 7 days at 1% O_2 and 5% CO_2. After 3 days of exposure to test agents, *M. tuberculosis* in each well was diluted 5-fold by the addition of 60 μL of fresh replicating medium using a reagent dispenser (ThermoScientific), which also served to mix cells. After 7 days of outgrowth at 20% O_2 and 5% CO_2, the OD_{580} was determined. Primary screening hits and downstream assay data were managed using the CDD Vault from Collaborative Drug Discovery (Burlingame, CA. www.collaborativedrug.com)[58] and JChem for Excel and MarvinView (ChemAxon).

Activity against Replicating and Nonreplicating *M. tuberculosis*. For minimal inhibitory concentration (MIC) assays, compounds were serially diluted 2-fold in DMSO from 10 to 0.04 mM using a PerkinElmer Janus robot with a P30 row/column MDT head to make 100× compound source stocks in Greiner compound plates (384-well small volume conical well, reference number 784201). Compounds were then distributed into 384-well replicating and nonreplicating assays with *M. tuberculosis* mc^{2}6220 in 384-well microplates as described above. For colony forming unit assays, experiments were set up using wild-type *M. tuberculosis* single cell suspensions in 96-well tissue culture-treated plates (Corning). At select time points, aliquots of cells were serially diluted in PBS-Tyl and spread on Middlebrook 7H11 agar plates containing a 10% OADC supplement. Colonies were enumerated ∼3 weeks postplating. The minimal bactericidal concentration leading to 99% reduction in colony forming units (MBC_{99}) was extrapolated from CFU data.

HepG2 Toxicity Assays. Toxicity assays using the human hepatoma cell line HepG2 were as described.[59] Briefly, HepG2 cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), pyruvate, glutamine, and

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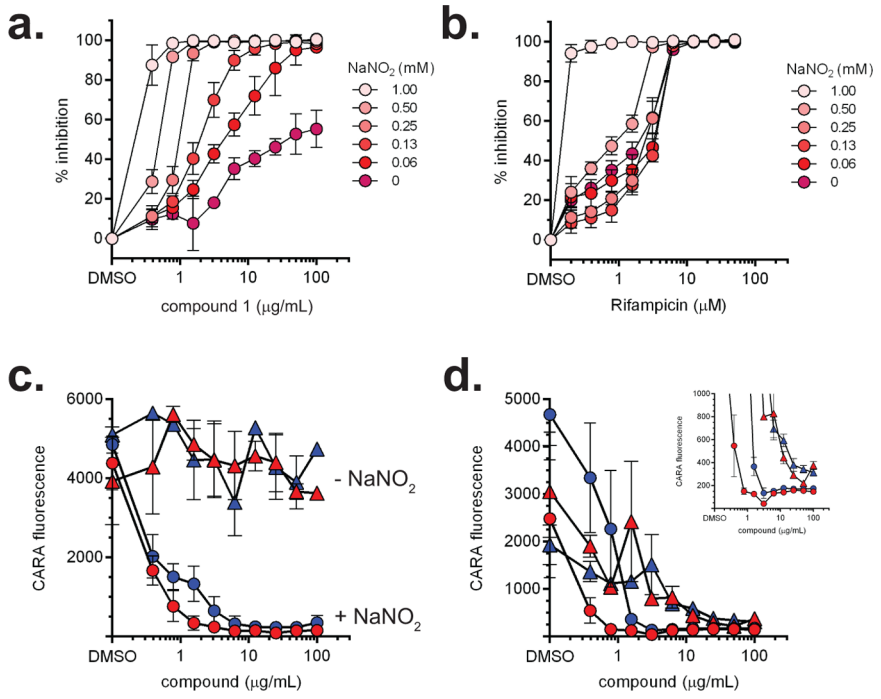

Figure 6. Potentiation of activity of cephalosporins against nonreplicating *M. tuberculosis* by reactive nitrogen species. Wild-type *M. tuberculosis* was resuspended at an OD_{580} of 0.1 in nonreplicating medium containing indicated concentrations of $NaNO_2$ (0−1 mM) and dispensed into separate microtiter plates for each $NaNO_2$ concentration. Cells were then exposed to (a) **1** or (b) rifampicin for 7 days, after which a standard outgrowth assay was initiated to estimate the number of surviving cells. In a separate experiment, nonreplicating *M. tuberculosis* at a standard OD_{580} of (c) 0.1 or lower inoculum of OD_{580} of (d) 0.01 were treated with either **1** (red) or **5** (blue) in the presence or absence of 0.5 mM $NaNO_2$ for 7 days. CARA fluorescence provides an estimate of mycobacterial viability; complete loss of fluorescence is associated with ≥2−3 log_{10} CFU reduction.

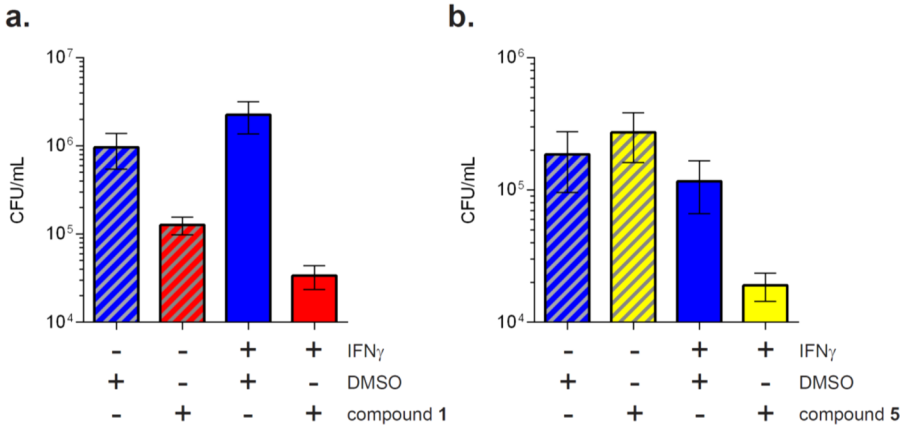

Figure 7. Bactericidal activity of (a) **1** and (b) **5** against intracellular *M. tuberculosis*. Mouse bone marrow-derived macrophages activated or not with 50 ng/mL of IFNγ were infected with wild-type *M. tuberculosis*. After a 4 h period for bacterial uptake, macrophages were washed and treated with 100 μg/mL of **1** or **5** for (a) 4 or (b) 3 days. Morphology of the macrophages was not affected by addition of **1** or **5** at the concentrations shown. One of five similar experiments.

nonessential amino acids. HepG2 cells were incubated for 2 days with DMSO vehicle control or test compounds (≤1% DMSO final) at 3000 cells/well in 384-well tissue culture plates (Greiner reference 781091). Cellular viability was determined after 2 days by measuring ATP content with a CellTiter-Glo kit (Promega).

Microbial Spectrum. Select compounds were tested for activity against a panel of replicating Gram positive and Gram negative bacteria (*Mycobacterium smegmatis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and yeast (*Candida albicans*). Bacteriologic medium and assay conditions were as described.[21] In brief, 200 μL of cells at an OD_{580} of 0.01 in a sterile, clear tissue culture-treated Corning 96-well plate were exposed to DMSO or drug, and growth was determined by optical density.

Stability Assay. Compounds were dissolved at 50 μg/mL in cell-free PBS (pH 7.4) or cell-free nonreplicating medium (pH 5.0) containing or not 0.5 mM $NaNO_2$. Poorly soluble compounds were dissolved at 5 μg/mL and in a 50:50 (vol:vol) solution of acetonitrile and PBS, or acetonitrile and nonreplicating medium containing or not 0.5 mM $NaNO_2$. The nonreplicating medium was as described above except that BSA, tyloxapol, lysine, and pantothenate were omitted. Solutions containing acetonitrile had their pH adjusted to 5.0 (the additional acetonitrile increased the pH from 5.0 to 5.8). Samples were incubated at 37 °C, and aliquots were removed every 12 h for analysis by LC-MS. Data represent percent remaining of the parent compound compared to that at the start of the experiment.

Cheminformatics. Tanimoto similarity between molecules and cheminformatic analysis of chemical properties (including ClogP

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values) were determined in Collaborative Drug Discovery (CCD) (Burlingame, CA; www.collaborativedrug.com)[58] using ChemAxon software.

Physicochemical, Permeability, and Metabolism Studies. These assays were performed by BioDuro (Shanghai, China). Mouse and human liver microsomal stability was assayed in triplicate at 0, 15, 30, 45, and 60 min.

Plasma Stability. Cephalosporins **1**, **5**, and cephalexin were spiked into lithium heparin-treated human and CD-1 mouse plasma (bioreclammation) at 1 μg/mL. Spiked samples were incubated at 37 °C, and extractions were performed at 5, 15, 30, 60, and 180 min. The reactions were quenched and proteins precipitated at each time point by adding 20 μL of spiked plasma to 200 μL of extraction solvent containing methanol/acetonitrile (1:1; vol/vol) and 10 ng/mL of verapamil (Toronto Research Chemicals, Inc.) as an internal standard (IS). In addition, 20 μL of 1:1 acetonitrile/water (ACN/H_2O; vol/vol) were added. A reference sample was created by adding 20 μL of unspiked plasma to 200 μL of the extraction solvent. After the plasma enzymes were denatured by the extraction solvent, 20 μL of a 1 μg/mL solution in 1:1 ACN/H_2O was added to the reference sample. Extracted samples were vortexed for 5 min and then centrifuged at 3000 rpm for 5 min. Then, 100 μL of extract was transferred to 100 μL of ddH_20 for LC-MS analysis. LC-MS analysis was performed with an Agilent 1260 liquid chromatography system coupled to a 4000 Qtrap mass spectrometer (AB Sciex) in multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI) and an Agilent column, SB-C8, 2.1 × 30 mm, 3.5 μm. Mobile phase A was 0.1% formic acid in 100% H_2O, and mobile phase B was 0.1% formic acid in 100% acetonitrile. Injection volumes were routinely 2 μL. The ions monitored were compound **1** (m/z 387.1/195.9), compound **5** (391.1/199.9), cephalexin (348.1/158.1), and verapamil (455.4/165.2). The percentage remaining was determined at each time point by dividing the sample analyte/IS peak area ratio by the reference sample analyte/IS peak area ratio.

Charcoal Agar Resazurin Assay (CARA). The CARA was used as described.[42] In brief, 10 μL aliquots from replicating or nonreplicating MIC_{90} assay plates were removed and spotted onto microplates containing 200 μL of 7H11-OADC-charcoal agar in each well. The microplates were then incubated for 7−10 days at 37 °C at 20% O_2 and 5% CO_2. The film of bacterial growth (microcolonies) on the microplates was semiquantitated by the addition of 40 μL of a 1:1 (v/v) mixture of Alamar blue (AB) and Tween80 (TW80) and 1 h of further incubation at 37 °C at 20% O_2 and 5% CO_2. In some cases, if the CARA microplate appeared dry, all wells were prewetted with 40 μL of PBS prior to the addition of the AB:TW80 developing solution. Fluorescence was determined by top-read with excitation at 530 nm and emission at 590 nm. The CARA-minimal bactericidal concentration leading to ≥99% loss in CFUs (CARA-$MBC_{\geq 99}$) was estimated as the lowest concentration of drug leading to complete loss of Alamar blue fluorescence.

Macrophage Infections. Primary bone marrow-derived macrophage infections were performed as described.[17,60−62] In brief, ∼1 × 10^5 macrophages isolated from 8-week old female C57Bl6 mice were grown in 48-well plates in DMEM supplemented with 4.5 g/L of glucose, 0.584 g/L of L-glutamine, 1 mM pyruvate, 10% FBS, and 10% L-cell conditioned medium containing or not 50 ng/mL of recombinant mouse IFNγ and infected with wild-type *M. tuberculosis* H37Rv at a multiplicity of infection of 1−5. Log phase wild-type *M. tuberculosis* was allowed to infect macrophages for 4 h, after which medium and extracellular *M. tuberculosis* were removed by two washes with PBS and replaced with fresh medium containing compounds or not at 1% DMSO. At the times indicated, macrophages were washed and lysed with PBS supplemented with 0.5% Triton X-100. Surviving bacilli were enumerated on 7H11-OADC agar plates. Macrophage supernatants were assayed for nitrite with the Greiss assay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01833.

Experimental and analytical details for synthetic analogues and copies of ^1H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

¶Dedicated to the memory of Lester A. Mitscher

■ ABBREVIATIONS USED

AB, Alamar blue; ACN, acetonitrile; 7-ADCA, 7-aminodeacetoxycephalosporanic acid; CARA, charcoal agar resazurin assay (CFU-surrogate assay); CDD, collaborative drug discovery; CFU, colony-forming unit; DlaT, dihydrolipoamide acyltransferase; DMEM, Dulbecco's modified eagle medium; IFNγ, interferon γ; IS, internal standard; LDT, L,D-transpeptidase; MDT, modular dispense technology; MRM, multiple reaction monitoring; *Mtb*, *Mycobacterium tuberculosis*; NR, nonreplicating; OADC, oleic albumin dextrose catalase; PBS-Tyl, PBS-containing tyloxapol; R, replicating; RNS, reactive nitrogen species; TW80, Tween80

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