

# Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux

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Although the classical antibiotic spectinomycin is a potent bacterial protein synthesis inhibitor, poor antimycobacterial activity limits its clinical application for treating tuberculosis. Using structure-based design, we generated a new semisynthetic series of spectinomycin analogs with selective ribosomal inhibition and excellent narrow-spectrum antitubercular activity. In multiple murine infection models, these spectinamides were well tolerated, significantly reduced lung mycobacterial burden and increased survival. *In vitro* studies demonstrated a lack of cross resistance with existing tuberculosis therapeutics, activity against multidrug-resistant (MDR) and extensively drug-resistant tuberculosis and an excellent pharmacological profile. Key to their potent antitubercular properties was their structural modification to evade the Rv1258c efflux pump, which is upregulated in MDR strains and is implicated in macrophage-induced drug tolerance. The antitubercular efficacy of spectinamides demonstrates that synthetic modifications to classical antibiotics can overcome the challenge of intrinsic efflux pump-mediated resistance and expands opportunities for target-based tuberculosis drug discovery.

Nearly 70 years after the discovery of streptomycin, which was the first antibiotic used to treat tuberculosis, this disease remains a formidable cause of human mortality, resulting in 1.4 million deaths per year<sup>1</sup>. MDR strains cause an estimated 300,000 deaths per year, and extensively drug-resistant (XDR) tuberculosis has spread to 84 countries, with some strains being reportedly resistant to all available drugs<sup>2</sup>. With the exception of rifamycins, antitubercular therapeutic regimes consist of unaltered natural products or completely synthetic molecules. This is in contrast to the therapeutic regimes for other bacterial infections, which are dominated by semisynthetic derivatives of natural products. The success of semisynthetic drugs is attributable to the high structural diversity of their antibiotic cores that are not found in purely synthetic collections and the synthetic modifications that maximize potency, safety and distribution in humans<sup>3,4</sup>. With the expanding structural and molecular information now available for drug targets, reevaluation of existing antibacterial classes that have been underutilized for tuberculosis may provide opportunities for synthetic modifications that retain or improve target affinity while circumventing native resistance mechanisms, such as efflux.

One structurally distinct antibiotic that has not yielded any approved semisynthetic analogs and has limited activity against *Mycobacterium tuberculosis* is spectinomycin. Spectinomycin has been used historically as a second-line agent to treat gonorrheal infections. Although spectinomycin is chemically similar to aminoglycosides, it binds to a separate site within the 16S bacterial ribosomal subunit, designated helix 34, and blocks ribosome translocation<sup>5,6</sup>. Unlike aminoglycosides, spectinomycin has a high safety margin, with only minor side effects (injection site soreness, chills and nausea) and no nephrotoxicity or ototoxicity when administered for a short term at high therapeutic doses<sup>7,8</sup>.

In this study, we describe the design, synthesis and evaluation of a class of new semisynthetic spectinomycin analogs, the spectinamides, as antitubercular agents. These studies were inspired by (i) the finding that researchers in the 1980s were able to generate spectinomycin analogs with increased activity against gram-positive pathogens<sup>9–12</sup>; (ii) the crystal structures of spectinomycin bound to the ribosome that became available recently<sup>6</sup>; and (iii) the design approach used for tigecycline<sup>13</sup>, which is a semisynthetic broad-spectrum tetracycline

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Received 25 October 2013; accepted 19 December 2013; published online 26 January 2014; doi:10.1038/nm.3458

whose increased activity has been ascribed to efflux avoidance by the key synthetic addition of a glycol side chain<sup>14</sup>. From this knowledge, we hypothesized that a structure-based design cycle could be used to generate spectinomycin analogs that have increased ribosomal target affinity, avoid active efflux or both, thus gaining potency against *M. tuberculosis* at therapeutically achievable concentrations. The spectinamides that we describe here are potent bacterial ribosomal inhibitors that avoid efflux by *M. tuberculosis* to achieve excellent antitubercular efficacy *in vivo*.

## RESULTS

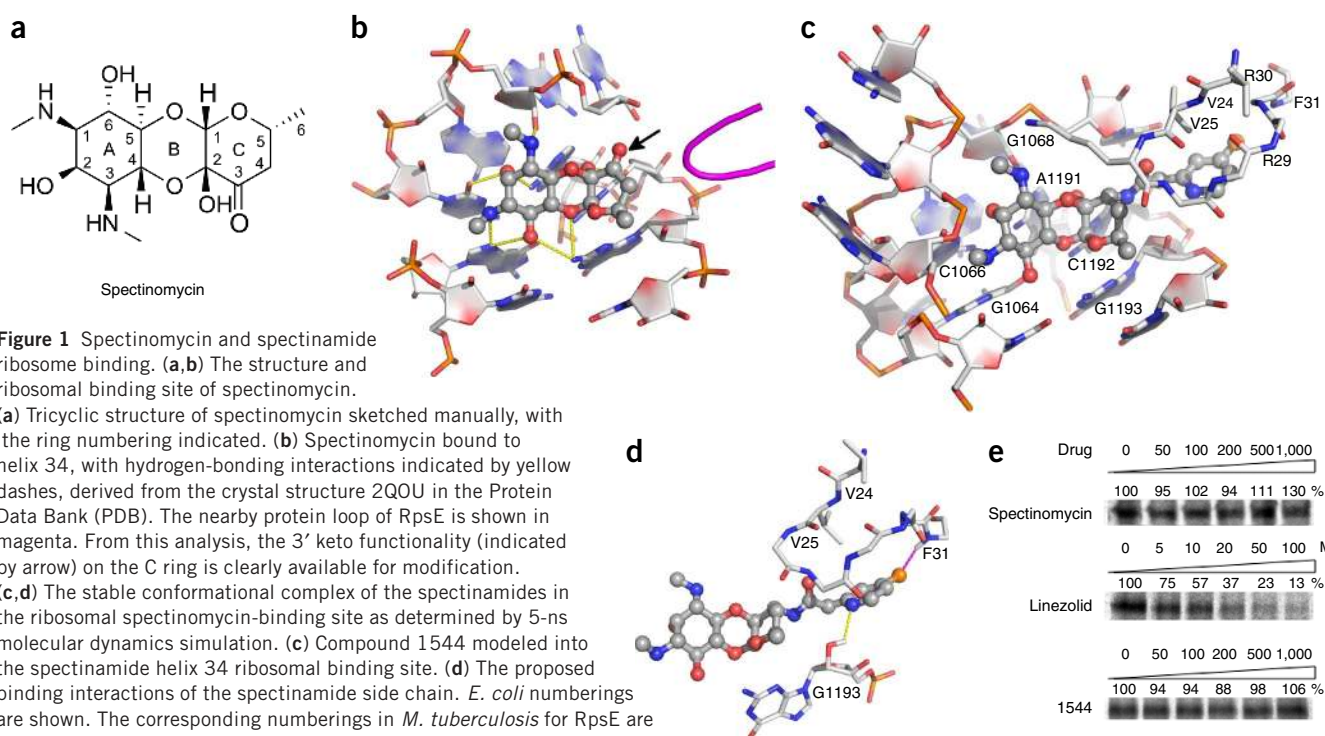
### Structure-guided modification improves activity

Spectinomycin has a uniquely fused tricyclic architecture in which the diaminocyclitol moiety actinamine (ring A) is fused to a single sugar component actinospectose (ring C) through a  $\beta$ -glycosidic and hemiketal linkage to form ring B (Fig. 1a)<sup>15</sup>. To determine how spectinomycin could be structurally modified while retaining ribosomal affinity, we analyzed past literature and recent structural studies and built a homology model of the *M. tuberculosis* 16S helix 34 spectinomycin-binding site from the *Escherichia coli* 30S spectinomycin structure<sup>6</sup>. These analyses revealed that any modification of the spectinomycin core was most likely not possible through the aminocyclitol A ring<sup>16–18</sup>, the oxocyclic B ring or the B-C ring fusion, which are responsible for most of the key hydrogen-bonding interactions within helix 34 of the 16S ribosomal RNA (Fig. 1b). However, stereospecific modification of the 3' keto group to an *R*-amine was well tolerated in our model predictions. From this, we hypothesized that derivatization of the amine would allow the introduction of functional groups that could both potentially make extra ribosomal contacts adjacent to helix 34 and with protein loop RpsE and modulate transporter affinity and overall physicochemical properties.

Consequently, we synthesized an initial panel of 16 substituted spectinamides and tested them for antitubercular potency, antibacterial spectrum of activity and protein synthesis inhibition (Supplementary Table 1). We achieved this synthesis in a convergent four-step sequence from spectinomycin<sup>10</sup> (Supplementary Scheme 1). From this compound set, we discovered 1329 as the initial lead (Table 1), which showed good minimal inhibitory concentration (MIC) activity that was specific to *M. tuberculosis* (1.6  $\mu\text{g ml}^{-1}$ ) and inhibition of mycobacterial ribosomal translation (1.2  $\mu\text{g ml}^{-1}$ ). Notably, several analogs in this compound set, such as 1351, inhibited mycobacterial ribosomes in *in vitro* translation assays at low concentrations (half-maximum inhibitory concentration (IC<sub>50</sub>) 0.37  $\mu\text{g ml}^{-1}$ ) but had poor antitubercular activity (25  $\mu\text{g ml}^{-1}$ ). These findings suggest that, similarly to spectinomycin, these compounds did not concentrate in the cell<sup>19</sup>, whereas 1329 accumulated intracellularly.

### Spectinamides benefit from additional ribosomal contacts

We further rationalized spectinamide binding by *in silico* docking into our active-site model using Glide<sup>20</sup> and performing 5-ns molecular dynamics simulations, which accounted for conformational flexibility within helix 34 and the nearby protein loop of RpsE. Molecular dynamics simulations suggested that lead spectinamides formed a stable complex in the spectinomycin-binding site, with the side chain making contacts in a previously unexplored pocket located adjacent to helix 34 and RpsE (Fig. 1c). This structure-based approach also helped to rationalize the structure-activity relationship (SAR) observed during development of the series: first, the need for a 2-pyridyl group that makes a hydrogen bond with the free ribosyl hydroxyls of C1192 or G1193 with an averaged calculated acceptor-donor distance of 3.04 Å; and second, the need for



**Figure 1** Spectinomycin and spectinamide ribosome binding. (**a,b**) The structure and ribosomal binding site of spectinomycin. (**a**) Tricyclic structure of spectinomycin sketched manually, with the ring numbering indicated. (**b**) Spectinomycin bound to helix 34, with hydrogen-bonding interactions indicated by yellow dashes, derived from the crystal structure 2QOU in the Protein Data Bank (PDB). The nearby protein loop of RpsE is shown in magenta. From this analysis, the 3' keto functionality (indicated by arrow) on the C ring is clearly available for modification. (**c,d**) The stable conformational complex of the spectinamides in the ribosomal spectinomycin-binding site as determined by 5-ns molecular dynamics simulation. (**c**) Compound 1544 modeled into the spectinamide helix 34 ribosomal binding site. (**d**) The proposed binding interactions of the spectinamide side chain. *E. coli* numberings are shown. The corresponding numberings in *M. tuberculosis* for RpsE are V55 (V24), V56 (V25), R60 (R29), R61 (R30) and F62 (F31) and for 16S are G1054 (G1064), C1056 (C1066), G1058 (G1068), A1182 (A1191), C1183 (C1192) and G1184 (G1193). (**e**) *In organello* mitochondrial expression of S<sup>35</sup> methionine-labeled cytochrome c oxidase subunit I (COX1) in the presence of inhibitors at the indicated doses as determined by densitometry of autoradiograms. Representative results from three independent experiments are shown.

**Table 1 Structures and activities of representative spectinamides**

Compound	MIC (wild type) <sup>a</sup> (μg ml <sup>-1</sup> )	MIC (ΔRv1258c) <sup>a</sup> (μg ml <sup>-1</sup> )	Mycobacterial ribosomes <sup>b</sup> (IC <sub>50</sub> , μg ml <sup>-1</sup> )	Mitohybrid ribosomes <sup>c</sup> (IC <sub>50</sub> , μg ml <sup>-1</sup> )	Cytohybrid ribosomes <sup>c</sup> (IC <sub>50</sub> , μg ml <sup>-1</sup> )	Rabbit reticulocyte ribosomes <sup>d</sup> (IC <sub>50</sub> , μg ml <sup>-1</sup> )	Cytotox IC <sub>50</sub> (μg ml <sup>-1</sup> ) <sup>e</sup>
1329	1.6	3.1	1.16	124.0	>500	>500	>200
1351	25	3.1	0.37	ND	ND	ND	>200
1398	25–50	0.8–1.6	3.10	ND	ND	ND	>200
1443	1.6	ND	0.65	119.0	>500	>500	>200
1445	0.8	0.8	0.55	66.6	>500	>500	>200
1544	0.8	0.8	0.54	49.8	>500	>500	>200
1599	1.6	ND	0.53	60.1	>500	ND	>200
Spec	50	6.2	0.39	>300	>500	>500	>200

<sup>a</sup>MIC activity against *M. tuberculosis* H37Rv parent and Rv1258c pump knockout strain (determined using method 1 described in the Online Methods). <sup>b</sup>Translation inhibition against *Mycobacterium smegmatis* (mycobacterial) ribosomes. <sup>c</sup>Bacterial ribosomes were mutated to contain the mitochondrial (mitohybrid) and cytosolic (cytohybrid) helix 34 sequences. <sup>d</sup>Rabbit reticulocyte ribosomes representing native eukaryotic ribosomes. <sup>e</sup>Mammalian cytotoxicity determined against the Vero and J774 cell lines. Spec, spectinomycin; ND, not determined.

an acetamide spacer that orientates the pyridyl ring appropriately to participate in these interactions.

Simulations indicated that the introduction of halogen substituents into the meta and para positions of the pyridyl ring of 1329 would increase complex stability by locking the pyridyl nitrogen hydrogen-bonding interaction with G1193 and forming favorable interactions with backbone amide groups in the RpsE loop (Fig. 1d)<sup>21</sup>. This structure-guided modification of 1329 successfully produced optimized leads (1445, 1544 and 1599) with improved antitubercular potency (Table 1).

### Spectinamides remain on target and lack cross resistance

All lead spectinamides retained activity against a panel of *M. tuberculosis* strains, including drug-susceptible strains, strains monoresistant to front-line therapeutics (Table 2) and MDR and XDR tuberculosis clinical isolates (Table 3 and Supplementary Tables 2 and 3). Spontaneous mutants of *M. tuberculosis* arose at the following frequencies: 1329 ( $1.9\text{--}3.7 \times 10^{-6}$ ), 1544 ( $1.3\text{--}3.1 \times 10^{-6}$ ) and 1599 ( $1.6\text{--}7.4 \times 10^{-7}$ ), which are comparable to the frequency for isoniazid ( $0.14\text{--}3.2 \times 10^{-7}$ ) and other front-line antitubercular drugs. Sequencing helix 34 in six spontaneous spectinamide-resistant mutants revealed changes at key nucleotides (C1066A, C1066G,

C1192A and A1191G), demonstrating that this modified series remained on the ribosome target<sup>22</sup>.

Notably, spectinamide-resistant mutants displayed no cross resistance with other protein synthesis inhibitors that are used to treat tuberculosis, including streptomycin, amikacin, kanamycin, capreomycin and linezolid. These results are consistent with the distinct ribosomal binding site for spectinomycin and its lack of inactivation by tubercular aminoglycoside-modifying enzymes such as acetyltransferase Eis<sup>23</sup>.

### Spectinamides are narrow-spectrum inhibitors active against nonreplicating *M. tuberculosis*

We saw substantial antibacterial activity only against our test set of mycobacteria from the tuberculosis complex and the closely related pathogens *Mycobacterium ulcerans* and *Mycobacterium marinum* (Supplementary Table 4) but not against an extensive panel of gram-positive and gram-negative bacteria (Supplementary Table 1). The only exception was activity we observed against an *E. coli* strain that is deficient in TolC, a component of the major efflux pump AcrAB-TolC (Supplementary Table 1). Subsequent analysis showed that lead spectinamides efficiently inhibit *E. coli* protein synthesis in coupled transcription-translation assays (Supplementary Fig. 1). As the spectinomycin-binding site is highly conserved across bacterial species, these results indicate active efflux or poor cellular uptake as a major cause of compound inactivity in other species.

We assessed the potential for spectinamides to act against latent *M. tuberculosis* infections by comparing the potency of 1599 to the front-line drugs isoniazid and rifampin under hypoxic conditions *in vitro*<sup>24</sup> (Supplementary Table 5). As we anticipated, a large proportion (>50%) of bacteria survived treatment with isoniazid, whereas less than 0.01% survived treatment with rifampin. Most encouragingly, 1599 was also highly active, as only 0.06% of bacteria survived treatment, indicating that spectinamides may be suitable to sterilize and act against persistent infections *in vivo*.

### Spectinamides overcome efflux-mediated intrinsic resistance

In *M. tuberculosis*, efflux pumps have been increasingly found to have a role in intrinsic resistance to many antimicrobial agents<sup>19</sup>. Recent studies demonstrated that deletion of the multidrug transporter

**Table 2 Activity of spectinamides against various monoresistant typed *M. tuberculosis* strains**

Strain	Resistance	MIC (μg ml <sup>-1</sup> )						
		1329	1443	1445	1544	1599	Spec	Strep
H37Rv	None	1.6	0.8	1.6	1.6	1.6	100	1.6
ATCC 35822 <sup>a</sup>	Isoniazid (katG S315T)	3.1	1.6	3.1	1.6	1.6	100	1.6
ATCC 35820	Streptomycin (rpsL K43R)	3.1	1.6	6.3	1.6	1.6	200	>200
ATCC 35838 <sup>b</sup>	Rifampin (rpoB S351L)	1.6	6.3	0.8	1.6	1.6	100	1.6
ATCC 35837 <sup>c</sup>	Ethambutol	3.1	1.6	6.3	1.6	1.6	100	1.6
H37Rv-HL-14 <sup>d</sup>	1329	>200	>200	>200	>200	>200	>200	1.6
H37Rv-HL-15 <sup>d</sup>	1329	>200	>200	>200	>200	>200	>200	1.6

<sup>a</sup>Isoniazid, MIC ≥ 12.5 μg ml<sup>-1</sup>. <sup>b</sup>Rifampin, MIC ≥ 25.0 μg ml<sup>-1</sup>. <sup>c</sup>Ethambutol, MIC ≥ 25.0 μg ml<sup>-1</sup>. <sup>d</sup>Representative spontaneous spectinamide-resistant mutants with base changes in 16S rRNA were not cross resistant to linezolid, capreomycin, streptomycin, kanamycin, isoniazid, ethambutol or rifampicin. MICs were determined using method 1 (Online Methods). Strep, streptomycin.

**Table 3 Activity of spectinamides against multiple isolates of MDR and XDR tuberculosis**

Compound	MIC <sub>90</sub> (μg ml <sup>-1</sup> ) <sup>a</sup>	MIC <sub>50</sub> (μg ml <sup>-1</sup> ) <sup>a</sup>	MIC range (μg ml <sup>-1</sup> ) <sup>b</sup>
1329	3.8	2.3	1.6–7.5
1445	3.8	1.6	0.8–3.8
1544	1.8	1.6	0.8–1.8
1599	1.9	0.8	0.3–1.9

The panel consisted of 19 MDR and 5 XDR strains; MDR strains were resistant to isoniazid, rifampicin and at least two other antitubercular antibiotics, whereas XDR strains were resistant to isoniazid, rifampicin, ofloxacin and kanamycin. All strains were resistant to at least four different classes of antimycobacterial drugs; this included eight streptomycin- and seven kanamycin-resistant strains.

<sup>a</sup>MIC<sub>90</sub> and MIC<sub>50</sub> refer to the MIC values at which 90% and 50% of the strains are susceptible. <sup>b</sup>The range refers to MIC values across the whole panel of 24 MDR and XDR strains. **Supplementary Table 3** lists additional details. MICs were determined using methods 1 and 2 (Online Methods).

Rv1258c reduces intrinsic spectinomycin resistance<sup>25</sup>. We suspected that this transporter could influence the activity of spectinamides, as 1351, a glycyl-substituted spectinamide, is a potent ribosomal inhibitor but is only weakly antitubercular, whereas 1329, a 2-pyridyl spectinamide, is a less potent ribosomal inhibitor but has much stronger antitubercular activity (**Supplementary Table 1**). These observations suggest that chemical variations on the spectinamide substituent could overcome efflux mechanisms in *M. tuberculosis*.

To further explore this idea, we tested spectinamides with good ribosomal inhibition and either excellent (1329, 1445 and 1544) or poor (1351 and 1398) antitubercular MICs against an Rv1258c-deficient strain of *M. tuberculosis*. We observed lower MIC values in the Rv1258c knockout mutant for 1351 and 1398, as well as for spectinomycin, but the potencies of 1329, 1445 and 1544 were unaffected (**Table 1**). Complementation of the knockout mutant with Rv1258c restored the MICs for 1351 and 1398 to wild-type levels (**Supplementary Table 6**), thus confirming the role of Rv1258c in their efflux. Inactivation of all other pumps tested did not affect the susceptibility to spectinomycin or the spectinamides tested (**Supplementary Table 6**).

### Spectinamides do not inhibit mammalian translation

There was no detectable cytotoxicity for the lead compounds against two mammalian cell lines (**Table 1**). Concerned with the close homology of bacterial and human mitochondrial ribosomes, the well-documented mitochondrial toxicities and consequent side effects of many antibacterial protein synthesis inhibitors<sup>26–29</sup>, we studied the effect of spectinamides on eukaryotic cytosolic and mitochondrial translation. In our *in organello* mitochondrial translation assay (**Fig. 1e**), linezolid had an IC<sub>50</sub> value of 13.5 μM, which is similar to previously reported values<sup>30</sup>. In contrast,

spectinomycin and 1544 did not inhibit *in organello* mitochondrial translation, even at concentrations up to 1 mM. To further validate target specificity and exclude off-target effects, we compared the selective activity of the compounds for bacterial and eukaryotic ribosomes in cell-free translation assays (**Table 1**)<sup>31,32</sup>. We tested the compounds first against rabbit reticulocyte ribosomes, which represent native eukaryotic ribosomes, and second against constructed bacterial hybrid ribosomes with the bacterial helix 34 drug-binding pocket replaced by the corresponding human mitochondrial and cytoribosomal homologs (**Supplementary Fig. 2**). Similarly to spectinomycin, the spectinamides showed excellent target selectivity by demonstrating antiribosomal activity that spared both the eukaryotic cytosolic ribosome (selectivity index ratio of IC<sub>50</sub> of the cytohybrid ribosome to IC<sub>50</sub> of the bacterial ribosome >500) and the eukaryotic mitochondrial ribosome (selectivity index ratio of IC<sub>50</sub> of the mitochondriahybrid ribosome to IC<sub>50</sub> of the bacterial ribosome >100) (**Table 1**). We performed an extensive *in vitro* profiling screen of leads 1544 and 1599 against 68 primary human molecular targets and the 5 major human cytochrome P450 enzymes to detect any relevant off-target pharmacologic effects (**Supplementary Table 7**). No off-target effects were observed, including no inhibition of CYP450 enzymes and no interaction with the cardiac hERG potassium ion channel.

### Lead spectinamides have favorable pharmacokinetic profiles

*In vitro* assays showed that the spectinamides had low plasma protein-binding properties and were stable against hepatic microsomal metabolism (**Table 4**). Pharmacokinetic studies in rats revealed extensive renal elimination in the unchanged form for all compounds, with a disposition pattern similar to that of other aminoglycoside antibiotics (**Table 4**). A pharmacokinetic study performed in mice for 1599 indicated similar dose-normalized systemic exposure between intravenous administration in rats and subcutaneous administration in mice, thereby bridging the results of the pharmacokinetic studies in rats to the subsequent *in vivo* efficacy studies in mice (**Supplementary Table 8**). The post-antibiotic effect of spectinamides ranged from moderate (15–27 h) for 1329, 1443 and 1445 to long (75–133 h) for the chlorinated compounds 1544 and 1599, with the post-antibiotic effect of 1599 being similar to that of streptomycin (132 h). In pharmacodynamic time-kill experiments, compound 1445 demonstrated time above MIC-dependent killing in contrast to the maximum concentration (C<sub>max</sub>)-dependent killing of aminoglycosides (**Supplementary Fig. 3**)<sup>33</sup>.

### Activity is sustained in multiple tuberculosis infection models

We performed initial evaluation of the compounds *in vivo* in an acute *M. tuberculosis* infection model to provide an assessment of drug

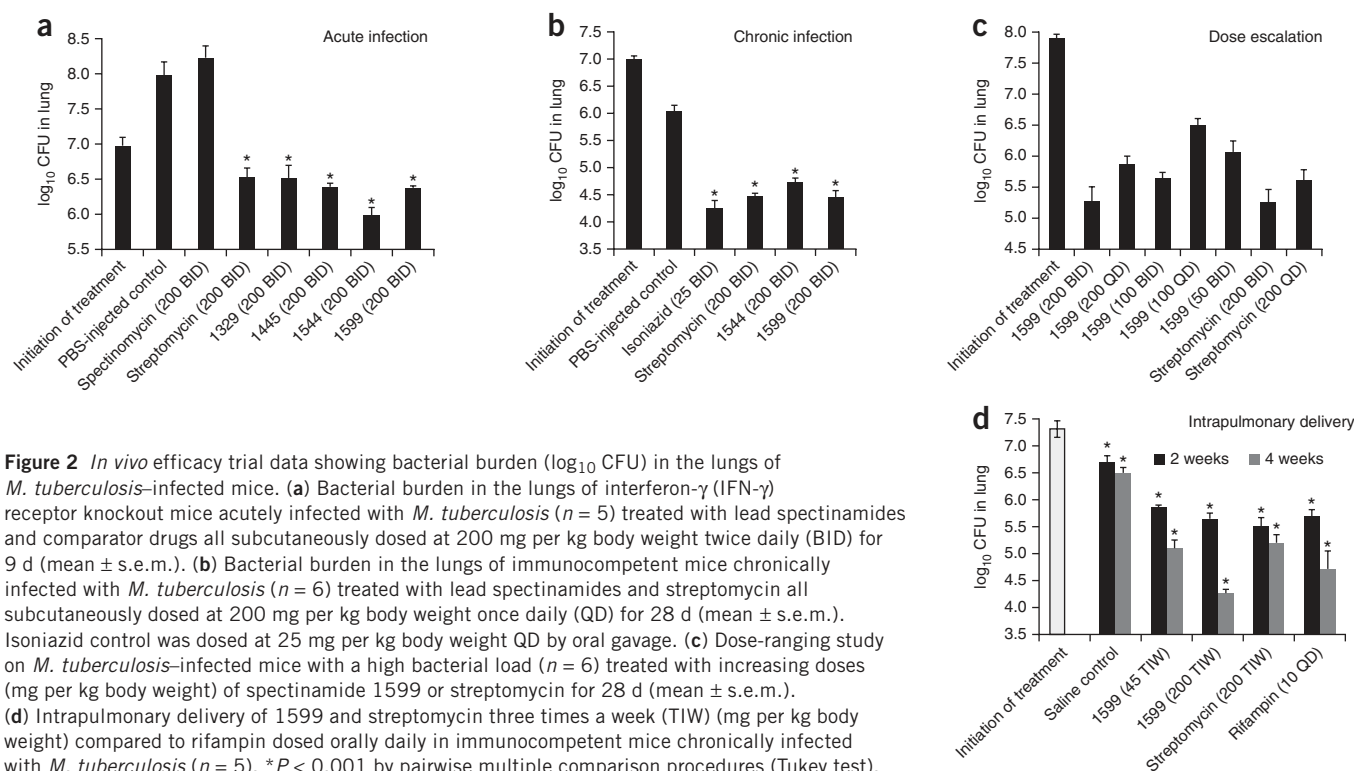
**Table 4 Pharmacokinetic and pharmacodynamic properties of lead spectinamides**

Property	Measurement	1329	1443	1445	1544	1599	Spectinomycin (unless noted)
Protein serum binding (rat)	Bound (%)	28.6 (8.9)	13.1 (0.3)	27.7 (4.3)	15.0 (9.9)	12.5 (1.7)	12.7 (7.5) <sup>b</sup>
Microsomal metabolic stability (rat)	Remaining after 90-min incubation (%)	83.7 (16.4)	109 (2.7)	102 (1.2)	67.3 (16.3)	88.0 (4.2)	80.4 (19.9)
i.v. pharmacokinetics (rat)	<i>t</i> <sub>1/2</sub> (h) <sup>a</sup>	0.52 (6.8)	0.44 (0.8)	0.45 (7.7)	1.06 (9.2)	0.58 (20.8)	0.75 (49.3) <sup>b</sup>
	Vd (l kg <sup>-1</sup> )	1.15 (11.9)	0.46 (10.3)	0.57 (7.7)	0.87 (22.9)	0.82 (34.5)	0.76 (45.2) <sup>b</sup>
	CL (l h <sup>-1</sup> kg <sup>-1</sup> )	0.72 (10.8)	0.89 (6.8)	0.56 (8.9)	0.94 (6.3)	1.22 (14.3)	0.60 (11.5) <sup>b</sup>
	Excreted unchanged in urine (%)	45.6 (9.7)	86.9 (12.5)	108 (8.3)	51.8 (9.9)	88.5 (11.5)	55.3 (27.0) <sup>b</sup>
Post-antibiotic effect	at ten times MIC (h)	18.8 (7.9)	26.9 (48.9)	15.7 (22.5)	75.4 (38.4)	133 (19.6)	Strep 132

All values are shown as the means (% coefficient of variation). All lead spectinamides had excellent aqueous solubility at >1 mM.

<sup>a</sup>*t*<sub>1/2</sub> is based on the decline of plasma concentration in the therapeutically relevant concentration range. <sup>b</sup>Indicates values published in ref. 44, which are included for comparison. i.v., intravenous; *t*<sub>1/2</sub>, half life; Vd, volume of distribution; CL, clearance.





**Figure 2** *In vivo* efficacy trial data showing bacterial burden (log<sub>10</sub> CFU) in the lungs of *M. tuberculosis*-infected mice. (a) Bacterial burden in the lungs of interferon- $\gamma$  (IFN- $\gamma$ ) receptor knockout mice acutely infected with *M. tuberculosis* ( $n = 5$ ) treated with lead spectinamides and comparator drugs all subcutaneously dosed at 200 mg per kg body weight twice daily (BID) for 9 d (mean  $\pm$  s.e.m.). (b) Bacterial burden in the lungs of immunocompetent mice chronically infected with *M. tuberculosis* ( $n = 6$ ) treated with lead spectinamides and streptomycin all subcutaneously dosed at 200 mg per kg body weight once daily (QD) for 28 d (mean  $\pm$  s.e.m.). Isoniazid control was dosed at 25 mg per kg body weight QD by oral gavage. (c) Dose-ranging study on *M. tuberculosis*-infected mice with a high bacterial load ( $n = 6$ ) treated with increasing doses (mg per kg body weight) of spectinamide 1599 or streptomycin for 28 d (mean  $\pm$  s.e.m.). (d) Intrapulmonary delivery of 1599 and streptomycin three times a week (TIW) (mg per kg body weight) compared to rifampin dosed orally daily in immunocompetent mice chronically infected with *M. tuberculosis* ( $n = 5$ ). \* $P < 0.001$  by pairwise multiple comparison procedures (Tukey test).

efficacy primarily against rapidly replicating bacteria (Fig. 2a and Supplementary Table 9)<sup>34</sup>. The four lead compounds (1329, 1445, 1544 and 1599) demonstrated significant *in vivo* efficacy in reducing the bacterial burden in lungs when compared to saline controls ( $P < 0.001$ ), showing more than one log<sub>10</sub> colony forming unit (CFU) reduction as compared to control after 9 consecutive days of treatment (Supplementary Table 9). There were no statistical differences between the activity of the spectinamide derivatives and streptomycin when administered at similar doses ( $P > 0.05$ ). We performed five independent acute infection mouse studies for 1544 and 1599, which all showed similar results. To assess activity against a chronic tuberculosis infection, we evaluated 1544 and 1599 in BALB/c mice after 28 days of treatment (Fig. 2b)<sup>35</sup>. Subcutaneous administration of 1544 or 1599 significantly decreased lung bacterial burden ( $P < 0.001$ ) (Supplementary Table 10) with no apparent toxicity. The activities of both compounds were similar to those of streptomycin ( $P > 0.05$ ) and isoniazid ( $P > 0.05$ ).

We then performed a dose-escalation efficacy trial with 1599 in BALB/c mice infected with a high and ultimately fatal tuberculosis burden (Fig. 2c). All untreated mice and mice receiving 1599 at 50 mg per kg body weight per day became moribund by day 18 and were humanely euthanized. The bacterial burden in the lungs of surviving mice showed a statistically significant reduction after drug treatment when compared to the start-of-treatment controls ( $P < 0.001$ ) (Fig. 2c and Supplementary Table 11). The efficacy of 1599 in this model was not statistically different from that of streptomycin at similar doses ( $P > 0.05$ ). We noted no apparent drug toxicity over the 28-day treatment period. Lead 1599 was recently included in one additional lethal bacterial burden and two additional chronic trials. In each trial, the results were similar to those presented in Figure 2b,c.

We also determined the efficacy of 1599 by intrapulmonary delivery in which we administered the compound intratracheally

to chronically infected mice using a liquid microsprayer (Fig. 2d). In this trial, 1599 demonstrated excellent efficacy in a dose-dependent manner. Treatment resulted in a 2.2 log CFU reduction in total lung bacterial burden as compared to the control group after 4 weeks of treatment at the highest dose (200 mg per kg body weight 3 days per week). The reduction in bacterial load by 1599 was comparable to that of rifampin ( $P > 0.05$ ) and was statistically superior to that of streptomycin delivered by a similar dose, route and schedule as 1599 ( $P = 0.004$  comparing the 1599 and streptomycin treatment groups) (Supplementary Table 12). 1599-treated mice had continued reduction in bacterial load in the lungs between weeks 2 and 4 of treatment ( $P < 0.05$ ), whereas we observed no additional killing for streptomycin after 2 weeks of treatment. In this trial, 1599 was well tolerated by intrapulmonary delivery, and we noted no apparent toxicity (no severe weight loss or death).

## DISCUSSION

The devastating socioeconomic and public health impact of tuberculosis, the emergence of MDR and XDR strains and the known toxicity of many existing antitubercular drugs all underscore the need for new drug candidates with excellent antitubercular activity and good safety profiles. Here we report the discovery of a new antitubercular spectinamide series generated by the synthetic modification of spectinomycin. We demonstrated that (i) the potency of this series is a product of high affinity for the mycobacterial ribosome and the avoidance of efflux; (ii) efflux-mediated drug resistance in *M. tuberculosis* can be overcome through chemical modification of the substrate antibiotic; and (iii) spectinamides are excellent preclinical drug candidates for tuberculosis with potent *in vivo* efficacy as well as a safe *in vitro* pharmacological profile.

Structural studies revealed a tight SAR for this series, with absolute need for an *R*-amide at the 3' position of the spectinamine

C ring coupled to a 2-pyridyl or 2-thiazole ring through an acetamide linker to maximize ribosomal affinity and, most importantly, avoid pump-mediated efflux. Compounds were further optimized by introducing halogen substituents to the 4 and 5 positions of the pyridyl ring, which, according to computational modeling, resulted in additional stabilizing interactions with backbone residues of the nearby RpsE protein loop<sup>36</sup>. The requirement for a 2-pyridyl or bioisostere 2-thiazole group appears to be twofold: (i) the ring nitrogen is well positioned to form a hydrogen bond with the free ribosyl hydroxyl group on G1193 in helix 34; and (ii) this nitrogen appears to be key to avoiding efflux, as the phenyl analog 1398 has poor MIC values despite good *in vitro* protein synthesis inhibition. One explanation for this requirement is that this heteroaryl nitrogen atom may allow for the formation of a favored intramolecular hydrogen bond in solution between it and the amide NH<sup>37</sup>, thus masking some of the molecule's polarity and helping the spectinamides avoid Rv1258c-mediated efflux.

The Rv1258c efflux pump (also known as the Tap efflux pump) is induced in macrophage-resident *M. tuberculosis* and may promote intracellular survival by allowing the organism to escape killing by the host immune response<sup>38</sup>. It is also responsible for the efflux of spectinomycin, resulting in high MICs that prevent its clinical utility<sup>19,25</sup>. We established that Rv1258c pump-mediated efflux can be avoided by structural modification of spectinomycin, thereby greatly reducing the MIC. This challenges the historical notion that *M. tuberculosis* is intrinsically resistant to many standard antibiotics because of its greasy coat<sup>39</sup>, which excludes polar antibiotic substances. It is becoming clear that intrinsic efflux transporters, such as Rv1258c, are important in reducing intracellular drug concentrations and may have an important role in persistence of *M. tuberculosis*<sup>25,38</sup>. Thus, compounds designed to avoid pump-mediated efflux may have superior activity against hard-to-kill, drug-tolerant bacteria, which may lead to better *in vivo* efficacy.

Microbial and pharmacological testing of the spectinamides has demonstrated that they possess many properties that are attractive for preclinical candidacy. First, spectinamides have a narrow spectrum of activity that is most likely associated with pump-mediated efflux, or possibly limited uptake, in other species. Therefore, long-term treatment of tuberculosis with spectinamides is unlikely to select for resistance in nontargeted pathogens. Second, the site of action of spectinamides within the ribosome is advantageous, as it does not overlap with other protein synthesis inhibitors, and drug binding is specific to the bacterial ribosome. This is evident in the lack of cross resistance with other clinically relevant protein synthesis inhibitors and in the pharmacology of the series. Studies demonstrated no appreciable cytotoxicity and no *in vitro* activity against eukaryotic cytosolic or mitochondrial ribosomal translation. This reduces the potential for side effects, such as ototoxicity<sup>27,40</sup> and myeloid suppression<sup>29,30</sup>, that are commonly associated with other protein synthesis inhibitors. In addition, potential concerns of drug-drug interactions during future use in combination pharmacotherapy regimens are reduced on the basis of *in vitro* metabolic profiling and pharmacokinetic studies. Extensive *in vitro* testing against mammalian enzymes and receptors showed that our spectinamide leads have little potential for off-target effects that result in adverse reactions, including those associated with drug metabolism and cardiac toxicity. Pharmacokinetic studies showed good distribution throughout the body, low plasma protein binding and elimination mediated largely by unchanged excretion into the urine. This is in contrast to most of the other new tuberculosis drug candidates that have been derived from

phenotypic screening, which tend to be highly lipophilic and highly protein bound. Thus, the spectinamides afford the possibility of reaching subpopulations that are unattainable by lipophilic antituberculosis agents<sup>41</sup>. These properties combined with the high safety index of the parent drug spectinomycin<sup>7,8</sup> suggest that the spectinamides will have a safe pharmacological profile that is favorable for further preclinical drug development.

Most notably, our lead compounds demonstrated potent antitubercular activity *in vivo* in multiple tuberculosis infection mouse models. Excellent antitubercular activity was observed in both acute and chronic tuberculosis models. In addition, twice-daily treatment with 1599 was equivalent to treatment with streptomycin in a model with high bacterial burden, rescuing mice from an otherwise lethal tuberculosis infection. Intrapulmonary administration of 1599 also provided excellent protection, reducing the bacterial load throughout the 4-week treatment period, suggesting sterilizing activity, whereas streptomycin apparently did not show further killing beyond the first 2 weeks of treatment. The excellent efficacy shown by 1599 after intrapulmonary delivery may reflect a good distribution of free drug within the lung coupled with its ability to avoid Rv1258c drug tolerance, which has been shown to be induced in alveolar macrophages<sup>38</sup>.

Our study supports the further development of spectinamides as new antitubercular agents owing to their tight SAR, a narrow antimicrobial spectrum, low synthesis costs and excellent *in vivo* efficacy and *in vitro* safety profiles. The potential safety and efficacy of this series are major advancements over most second-line agents that are used to treat MDR tuberculosis. Widespread use of second-line drugs such as aminoglycosides has been limited because of their renal and auditory toxicities, as well as their narrow therapeutic window. Resistance to most second-line agents has also emerged, warranting the development of other therapeutic options. Further, spectinamides overcome clearance by the efflux pump Rv1258c, which has been implicated in phenotypic drug tolerance and is often upregulated in MDR tuberculosis strains<sup>38,42,43</sup>. These benefits and the demonstrated potency using intrapulmonary delivery for this class of compounds overshadow the general lack of oral bioavailability of aminocyclitols (including the spectinamides), which is a common limitation of many second-line agents forming the core of MDR and XDR therapy. This study represents the first steps toward developing an antitubercular drug class using a semisynthetic approach to overcome intrinsic pump-mediated resistance mechanisms. Studies toward the clinical development of spectinamides as antitubercular agents are ongoing.

## METHODS

Methods and any associated references are available in the [online version of the paper](#).

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

## ACKNOWLEDGMENTS

This study was supported by US National Institutes of Health (NIH) grant AI090810 (R.E.L., E.C.B., A.J.L. and B.M.), the National Institute of Allergy and Infectious Diseases (NIAID) Indefinite Delivery Indefinite Quantity (IDIQ) (R.E.L. and M.G.) Contract Task Order HHSN2722010000091/01, the American Lebanese Syrian Associated Charities (ALSAC) and St. Jude Children's Research Hospital (SJCRH) (R.E.L.) and in part by the Intramural Research Program of the NIAID, NIH (H.I.B.) and the Spanish Government (grant BIO-2009-09405) (J.A.A.). We thank L. Yang and J. Scarborough from SJCRH for their help with analysis of the final compounds, M. Maddox from SJCRH for technical assistance in determining MIC values, J. Ryman from the University of Tennessee Health Science Center for technical assistance in the performance of pharmacokinetic

studies in mice, M. Butler from Microbiotix for coordination of the MDR tuberculosis testing and E. Tuomanen from SJCRH for critical evaluation of this manuscript.

#### AUTHOR CONTRIBUTIONS

R.E.L. designed the compound series. J.G.H., D.F.B., R.B.L. and H.I.B. performed MIC testing and microbiology studies. J.L., J.Q., R., S.L.W. and D.S. performed the medicinal chemistry. T.M., R.A. and E.C.B. designed and performed MIC testing and ribosome inhibition studies. M.S.S., M.R.M., M.G.-J. and A.J.L. designed and performed the *in vivo* efficacy trials. P.K.V., C.R., D.B.M., A.T. and B.M. designed and performed the pharmacokinetic analysis. Z.Z. and S.D. performed the molecular modeling experiments. C.V., D.F.B. and J.A.A. designed and performed the efflux mutant testing. All authors discussed and analyzed the data. R.E.L., E.C.B., A.J.L., R.B.L., D.F.B., J.A.A. and B.M. wrote the manuscript.

#### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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## ONLINE METHODS

**Molecular modeling.** A homology model of the *M. tuberculosis* 16S helix 34 spectinomycin-binding site was built using the crystal structure of *E. coli* in complex with spectinomycin (PDB 2QOU)<sup>6</sup>. To reduce the computational cost, an ~15-Å truncated sphere centered on the spectinomycin-binding site was used throughout the simulation. Residues including a single nucleotide of helix 34 and two residues of the RpsE protein loop were altered to correspond to the *M. tuberculosis* sequence (A1081G, T24V and I30R) while contacts within a 5-Å radius of spectinomycin were fully conserved. Terminal residues were harmonically restrained to their X-ray positions. The spectinamide series was sketched manually, and each geometry was optimized in Jaguar, version 7.8 (Schrodinger, LLC, Portland, OR) at the B3LYP/6-31G\*\* level of approximation. The compounds were then docked into the binding active site using Glide<sup>20</sup>, and top poses were retained. Molecular dynamics calculations were carried out with the AMBER11 program<sup>45</sup> using a FF03 force field for the RNA and protein and a General Amber Force Field for the ligand. The whole complex was solvated in an octahedron box of TIP3P water molecules and neutralized by adding sodium ions while keeping crystal ions. Periodic boundary conditions, particle-mesh Ewald treatment of the long-term electrostatics and SHAKE-enabled 2-fs time steps were employed. A two-stage energy minimization was performed, followed by a gradual heating of the complex system from 0 K to 300 K over 60 ps and a 50-ps equilibration. An additional 0.5-ns simulation at 300 K was performed to further optimize the system. All production runs were performed with the NPT ensemble for 5 ns.

**General methods to synthesize spectinamides.** The spectinamides were synthesized from spectinomycin (Waterstone Technology, IN) according to the procedure of Woitun<sup>10</sup>, except that the final deprotection step was modified using either catalytic hydrogenation and palladium on carbon (10% Pd/C) in 1.25 M HCl in methanol for 2 h or acid hydrolysis (compounds with hydrogenation-sensitive side chains) by reaction with 48% HBr solution at room temperature for 2 h (Supplementary Analytical Data).

**MIC and cytotoxicity testing.** MICs for aerobic bacteria were performed according to Clinical Laboratory Standards Institute (CLSI) methods, as described previously<sup>46,47</sup>. Cytotoxicity was determined as described<sup>48</sup> using Vero cells (ATCC CCL-81) and J774 cell lines and detection of viability with CellTiter96 assay (Promega).

***M. tuberculosis* susceptibility testing.** *Method 1.* Compounds were dissolved in 100% DMSO at a concentration of 10 mg ml<sup>-1</sup>. Twofold serial dilutions of compounds were prepared in 100 µl in of Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex and 0.05% (vol/vol) Tween 80. The optical density at 600 nm (OD<sub>600</sub>) of a mid log-phase *M. tuberculosis* culture was determined, and inoculum was prepared by adjusting the OD<sub>600</sub> to 0.01 in supplemented Middlebrook 7H9 broth. To each well of the 96-well round-bottom assay plate, 100 µl of inoculum was added to achieve a final drug concentration range of 200–0.2 µg ml<sup>-1</sup>, with no drug in column 12. Plates were placed in sealed bags and incubated at 37 °C for 7 d in ambient air, at which point the MIC was recorded as the lowest concentration of drug that prevented visible growth. In all assays, reference antibiotics (typically streptomycin and spectinomycin) were included for quality control.

*Method 2.* MICs were determined as described in method 1, except that the units of drug concentrations ranges were in molar concentrations. MIC values were then converted to units of µg ml<sup>-1</sup> for comparison with method 1 results.

*Method 3.* MICs were determined using an MGIT 960 equipped with TBeXiST software (Becton Dickinson) as described<sup>49</sup>.

**Drug testing using rapid anaerobic development model of dormancy.** 10-ml aliquots of *M. tuberculosis* H<sub>37</sub>Rv (diluted 1:100 from a mid log-phase culture) were delivered to glass culture tubes containing stir bars<sup>24</sup>. Tubes were capped with rubber septa and stirred rapidly (~800 r.p.m.) in a 37 °C incubator for 7 d, at which point the methylene blue indicator (1.5 µg ml<sup>-1</sup>) was reduced to yield a colorless culture. Using a sterile syringe, 100 µl of 1 mg ml<sup>-1</sup> test compound or carrier DMSO was injected through the septa. Cultures were incubated for an additional 7 d before enumeration of viable bacilli by titration of CFU. Results

from test cultures were normalized to the carrier-treated group. The results presented represent the average of two to three independent experiments.

**Construction of mutant ribosomes.** *M. smegmatis* mc<sup>2</sup>155  $\Delta$ rrnB $\Delta$ rrnAattB::rrnB was used for all genetic constructions<sup>31</sup>. Hybrid rRNA ribosomes (Supplementary Fig. 2) were constructed using PCR mutagenesis to generate mutant rDNA fragments that were cloned into the rRNA operon of integration-proficient plasmid pMV361 hyg-rrswt to result in plasmid pMV361 hyg-rrshybrid<sup>32</sup>. Plasmid pMV361 hyg-rrshybrid was transformed into *M. smegmatis*  $\Delta$ rrnB $\Delta$ rrnAattB::rrnB. Successful gene replacement of the wild-type rRNA operon by the hybrid rRNA operon through plasmid exchange was determined by sequence analysis. Ribosomes were purified from bacterial cell pellets as previously described<sup>50</sup>.

**Cell-free luciferase translation assays.** Purified 70S hybrid bacterial ribosomes and rabbit reticulocyte lysate (RRL, Promega) were used in translation reactions. Firefly luciferase mRNA was produced *in vitro* using T7 RNA polymerase. Translation reactions were carried out as previously described<sup>50</sup>. The IC<sub>50</sub> values represent the drug concentration that inhibits luciferase activity by 50%.

**Mitochondrial *in organello* translation.** Mitochondria were isolated from HEK293 cells<sup>51</sup>, and *in organello* translation was done as described with slight modifications<sup>30,52,53</sup>. In brief, the mitochondria-enriched pellet was resuspended in 1 ml of mitochondria reaction buffer containing cycloheximide (100 mg l<sup>-1</sup>) to inhibit cytosolic translation and S35-methionine (15 µl; 370 MBq (10 mCi) ml<sup>-1</sup>, specific activity 37TBq (1,000 Ci) mmol<sup>-1</sup>; Hartmann Analytic KSM-01) to label proteins synthesized by the mitochondrial ribosome. The suspension was split into equal aliquots of 54 µl, and drugs were added to a final reaction volume of 60 µl. Reaction mixtures were incubated for 2 h at 30 °C with shaking and centrifuged for 5 min at 15,000g, and the pellets were washed and resuspended in H<sub>2</sub>O. S35-methionine-labeled proteins were resolved by 18% SDS-PAGE. COX1 was identified by autoradiography on the basis of its molecular weight of 39 kDa and by the absence of the corresponding band when we used COX1 nonsense mutant cells<sup>54</sup> in *in organello* translation. Bands were quantified using an Aida Image Analyzer (Fuji). The IC<sub>50</sub> values represent the drug concentration that inhibits COX1 synthesis by 50%. Every experiment was repeated three times.

**Post-antibiotic effect (PAE).** PAE studies were performed in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), as described previously<sup>47</sup>. The difference between the time required to reach 50% saturation at OD<sub>600</sub> (1.1 ± 0.2) for the untreated and treated cultures was calculated as the PAE. Each treatment was tested in at least two biologically independent experiments.

**Protein binding.** Rat plasma protein binding was determined at 500 ng ml<sup>-1</sup> and 5 µg ml<sup>-1</sup> by equilibrium dialysis at 37 °C using the RED device (Thermo Scientific, Rockford, IL), as described previously<sup>55</sup>. Pre- and post-dialysis sample concentrations were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The average results of triplicate reactions from a single experiment are presented.

***In vitro* microsomal metabolic stability.** *In vitro* microsomal metabolic stability was assessed in pooled rat liver microsomal preparations (Cellzdirect, Austin, TX) according to the manufacturer's protocol. Disappearance of the parent compound was monitored by sampling at 0, 15, 30, 45, 60, 75 and 90 min during the incubation period and subsequent LC-MS/MS analysis<sup>55</sup>. The average results of triplicate reactions from a single experiment are presented.

**Pharmacokinetic studies.** The *in vivo* pharmacokinetic studies presented were performed at the University of Tennessee Health Science Center following protocol number 13-4263A, approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Compounds were administered to groups of catheterized (jugular and femoral veins) male Sprague-Dawley rats (8–10 weeks old, 200–225 g, *n* = 4–5) intravenously at a dose of 10 mg per kg body weight. Thirteen serial blood samples (~250 µl) were collected at predefined time points for 48 h after dosing. Plasma was separated immediately by centrifugation (9,300g for 10 min at 4 °C). Cumulative urine



samples were collected for 48 h. For pharmacokinetic bridging studies in mice, male C57BL/6 mice (8–9 weeks old, ~25 g,  $n = 24$ ) were dosed subcutaneously at 200 mg per kg body weight. Blood samples were collected by cardiac puncture before dosing and at seven predefined time points after dosing. Plasma was separated immediately by centrifugation as described above and stored at  $-80^{\circ}\text{C}$  until analysis. Compound concentrations in plasma and urine were quantified by LC-MS/MS analysis. The pharmacokinetic profiles of the compounds were analyzed by standard noncompartmental procedures<sup>55</sup> using Phoenix WinNonlin 6.2 (Pharsight Corporation, Mountain View, CA).

**Quantification of compound concentrations by LC-MS/MS.** 50- $\mu\text{l}$  aliquots of plasma and urine samples were prepared by protein precipitation with 200  $\mu\text{l}$  methanol (spiked with the internal standard 3'-dihydro-3'-deoxy-3'(R)-isopropylacetylaminospectinomycin) followed by centrifugation at 10,000g for 10 min at  $4^{\circ}\text{C}$ . Chromatographic separation of the supernatant was carried out on a Luna 3  $\mu\text{M}$  hydrophobic interaction liquid chromatograph (HILIC) 100  $\times$  4.6 mm column (Phenomenex, Torrance, CA) using a gradient mobile phase of methanol and 10 mM ammonium formate, pH 2.75, at a flow rate of 0.4 ml min<sup>-1</sup>. Detection was performed with an API 3000 triple-quadrupole mass spectrometer (ABI-Sciex, Foster City, CA) with electrospray ionization in multiple reaction monitoring mode using the compound-specific mass transfers of  $m/z$  453.3 $\rightarrow$ 247.3 for 1329,  $m/z$  459.2 $\rightarrow$ 207.2 for 1443,  $m/z$  471.3/207.1 for 1445,  $m/z$  487.2/128.3 for 1544,  $m/z$  487.2/207.1 for 1599 and  $m/z$  418.3/207.1 for 1369. A calibration curve ranging from 1.95 to 5,000  $\mu\text{g l}^{-1}$  was constructed for each test compound and validated with spiked samples of rat or mouse plasma or urine. The peak area ratios of analyte to the internal standard were linear over the concentration range tested for all compounds, with all correlation coefficients (weighted least-square linear regression analyses)  $>0.997$ . Accuracy (deviation of the analyzed quality-control samples from nominal values) was within  $\pm 3\%$  over the entire range of the calibration curve, and the precision (coefficient of variation of repeated measurements of the quality-control samples) was  $<2\%$  for all compounds.

**Pharmacodynamic time-kill studies.** Using a previously reported *in vitro* pharmacokinetic-pharmacodynamic (PK-PD) model system<sup>56</sup>, time-kill experiments were performed on *M. bovis* BCG Pasteur cultures in the presence of 1445. Total daily dose simulations (0.4, 2, 10 or 50 mg per kg body weight per day) were administered once daily, twice daily or three times daily. The number of viable bacteria in each sample was quantified by luminescence (BacTiter-Glo Promega, Madison, WI) and by standard CFU enumeration.

**In vivo efficacy of spectinamides in four mouse tuberculosis aerosol infection models.** All animal efficacy studies were performed at Colorado State University according to guidelines of the Colorado State University Institutional Animal Care and Use Committee and were approved under protocol number 13-4263A. For all mouse infection models, mice (5–6 per group) were challenged with an aerosol of *M. tuberculosis* Erdman (TMC 107, ATCC 35801) using a GlasCol aerosol chamber in a certified ABSL-3 laboratory according to guidelines of the Institutional Animal Care and Use Committee. Drugs were delivered by intrapulmonary administration in a 50  $\mu\text{l}$  volume or by subcutaneous injection in a 200  $\mu\text{l}$  volume. Streptomycin sulfate was formulated in phosphate buffered saline 1X for experiments in model 1 and 2, and in Plasma-Lyte A pH 7.4 for the model 3. Spectinamide trihydrochloride salts were used in model 1 and 2 experiments and consequently formulated in high-salt buffer (156 mM sodium phosphate and 137 mM NaCl), which was required to increase the pH to a final range of 6.5–7.5. Spectinamide dihydrochloride salts that required less buffering were used in the later experiments. In model 3, these salts were formulated in 1X Plasma-Lyte A pH 7.4 diluted in a ratio of 5 parts and 1 part sterile deionized water to maintain ideal osmotolerance. In model 4 the dihydrochloride spectinamide salts and streptomycin sulphate were formulated in endotoxin-free 0.9% saline for intratracheal administration. For endpoint analysis, mice were euthanized, and the lungs were collected. The left lung lobe was homogenized for enumeration of CFU by plating dilutions of the organ homogenates on nonselective 7H11 agar plates<sup>34</sup>. The CFU were converted to logarithms, which were then evaluated by a one-way analysis of variance (ANOVA) followed by a multiple-comparison ANOVA between all

mouse groups (to include control and treatment groups) by a one-way Tukey test (SigmaPlot, Systat Inc. San Jose, CA). For the high-burden infection trial, multiple-comparison ANOVA was conducted by Tukey test between treatment groups only, as no control mice survived without treatment intervention. Differences were considered significant at the 95% level of confidence.

**Model 1.** Efficacy in an acute infection model was tested using low-dose aerosol (LDA; 100 CFU per mouse) in female IFN- $\gamma$  knockout 8- to 10-week-old mice (Jackson Laboratories, Bar Harbor, ME) as described<sup>34</sup>. Starting 13 d after LDA, mice received the drug (200 mg per kg body weight subcutaneously) twice daily 9 h apart for 9 consecutive days. Lungs were harvested at day 22 after LDA. The model yielded similar results in five independent experiments, and results from a representative experiment are presented.

**Model 2.** Evaluation of compounds in a chronic tuberculosis model was performed in wild-type female 8- to 10-week-old BALB/c mice (Charles River Labs, Wilmington, MA) infected with an LDA<sup>35,57–59</sup>. After 21 d, mice were treated daily (5 days a week) with 200 mg per kg body weight of the drug subcutaneously. After 28 d of treatment followed by 2 d of drug clearance, lungs were harvested.

**Model 3.** Efficacy in a lethal infection model was tested by establishing a high bacterial burden of  $10^8$  log CFU in the lungs before treatment. Female 8- to 10-week-old BALB/c mice were challenged with a high-dose aerosol inoculum (3.5 log<sub>10</sub> CFU per mouse), which results in death without treatment intervention at around 18–20 d after aerosol challenge. At 13 d after exposure, mice were dosed either daily or twice daily (5 days a week) for up to 28 d with 1599 at 12.5, 25, 50, 100 or 200 mg per kg body weight; streptomycin was used as the control and was used at 200 mg per kg body weight in the same manner. Mice that became moribund were (humanely) euthanized. For all remaining mice, lungs were harvested after 28 d of treatment followed by 2 d of drug clearance.

**Model 4.** To determine efficacy using intrapulmonary nebulized drug delivery, 8- to 10-week-old BALB/c female mice were infected with an LDA, as in the chronic tuberculosis model (model 2). At 24 d after aerosol exposure, mice received drug by oral gavage (rifampin, 10 mg per kg body weight daily 5 days a week) or intrapulmonary aerosol delivery (1599 at 45 or 200 mg per kg body weight; streptomycin at 200 mg per kg body weight; or saline control) three times a week as previously described<sup>60</sup>. Mice were anesthetized using isoflurane, and aerosols were delivered employing a Microsprayer (model IA-C; PennCentury, Philadelphia, PA) attached to an FMJ-250 high-pressure syringe device (PennCentury) in a volume of 50  $\mu\text{l}$  per dose, as described<sup>61–63</sup>. After 2 or 4 weeks of therapy, lungs were harvested without a drug-free interval.

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