ANTIBIOTICS

Aminomethyl spectinomycins as therapeutics for drug-resistant respiratory tract and sexually transmitted bacterial infections

David F. Bruhn,[1]* Samanthi L. Waidyarachchi,[1]* Dora B. Madhura,[2] Dimitri Shcherbakov,[3] Zhong Zheng,[1] Jiuyu Liu,[1] Yasser M. Abdelrahman,[4,5] Aman P. Singh,[1,6] Stefan Duscha,[3] Chetan Rathi,[2] Robin B. Lee,[1] Robert J. Belland,[4] Bernd Meibohm,[2] Jason W. Rosch,[7] Erik C. Böttger,[3] Richard E. Lee[1]*†

The antibiotic spectinomycin is a potent inhibitor of bacterial protein synthesis with a unique mechanism of action and an excellent safety index, but it lacks antibacterial activity against most clinically important pathogens. A series of *N*-benzyl–substituted 3′-(*R*)-3′-aminomethyl-3′-hydroxy spectinomycins was developed on the basis of a computational analysis of the aminomethyl spectinomycin binding site and structure-guided synthesis. These compounds had ribosomal inhibition values comparable to spectinomycin but showed increased potency against the common respiratory tract pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, and *Moraxella catarrhalis*, as well as the sexually transmitted bacteria *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Non–ribosome-binding 3′-(*S*) isomers of the lead compounds demonstrated weak inhibitory activity in in vitro protein translation assays and poor antibacterial activity, indicating that the antibacterial activity of the series remains on target against the ribosome. Compounds also demonstrated no mammalian cytotoxicity, improved microsomal stability, and favorable pharmacokinetic properties in rats. The lead compound from the series exhibited excellent chemical stability superior to spectinomycin; no interaction with a panel of human receptors and drug metabolism enzymes, suggesting low potential for adverse reactions or drug-drug interactions in vivo; activity in vitro against a panel of penicillin-, macrolide-, and cephalosporin-resistant *S. pneumoniae* clinical isolates; and the ability to cure mice of fatal pneumococcal pneumonia and sepsis at a dose of 5 mg/kg. Together, these studies indicate that *N*-benzyl aminomethyl spectinomycins are suitable for further development to treat drug-resistant respiratory tract and sexually transmitted bacterial infections.

INTRODUCTION

The increasing prevalence of antibiotic resistance among common community- and hospital-acquired bacterial pathogens requires the discovery and development of new antimicrobials with unique mechanisms of action to treat infections caused by these organisms. Of major health concern are drug-resistant infections caused by cephalosporin-resistant *Neisseria gonorrhoeae* and multidrug-resistant respiratory pathogens including *Streptococcus pneumoniae* (*1*). The rise of these organisms comes at a time when the pipeline for the development of new antibiotics is not matching the increasing prevalence of drug-resistant organisms (*2*).

Synthetic modification of validated natural product scaffolds has been the most successful strategy in antibacterial drug discovery (*3*–*5*). This has led to the development of multiple generations of β-lactam, macrolide, and tetracycline antibiotics. Following this strategy, we have re-examined spectinomycin, the lowest–molecular weight member of the aminocyclitol family of antibiotics, which includes aminoglycosides (Fig. 1A). Spectinomycin binds selectively to a unique binding site in RNA helix 34 of the head domain of the 30S ribosomal subunit, blocking translocation and consequently protein synthesis (*6*–*8*). This binding site is distinct from that of other ribosomally active antibacterial therapeutics. Although spectinomycin is a potent inhibitor of bacterial protein synthesis in cell-free assays, it has only moderate antibacterial activity, limiting its current clinical application to drug-resistant gonorrhea. Spectinomycin's activity against *N. gonorrhoeae* is weak, yet the antibiotic's high safety margin permits use of 2- to 4-g intramuscular injections (*9*–*12*). Attempts to develop spectinomycin analogs in the 1980s led to the discovery of trospectomycin by researchers at UpJohn Company (Fig. 1B). Trospectomycin progressed to late-stage clinical trials for treatment of pelvic inflammatory disease (chlamydia) before being abandoned for commercial reasons, because the third-generation cephalosporins and second-generation macrolides in development and use were judged superior at the time. These efforts validate that modification of the spectinomycin core yields more potent generations of this antibiotic (*13*–*16*).

We have recently disclosed the discovery and preclinical advancement of a highly specific set of 3′-dihydro-3′-deoxy-(*R*)-acylamino spectinomycins (spectinamides, Fig. 1C), which are narrow-spectrum antitubercular agents active against multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis with robust activity in acute and chronic models of tuberculosis infection (*17*). Key to the antituberculosis activity of lead spectinamides was the introduction of

[1]Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, TN 38105, USA. [2]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN 38163, USA. [3]Institut für Medizinische Mikrobiologie, Universität Zürich, Zürich 8006, Switzerland. [4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN 38163, USA. [5]Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt. [6]Biomedical Sciences Graduate Program, University of Tennessee Health Science Center, Memphis, TN 38163, USA. [7]Infectious Diseases Department, St. Jude Children's Research Hospital, Memphis, TN 38105, USA.
*These authors contributed equally to this work.
†Corresponding author. E-mail: richard.lee@stjude.org

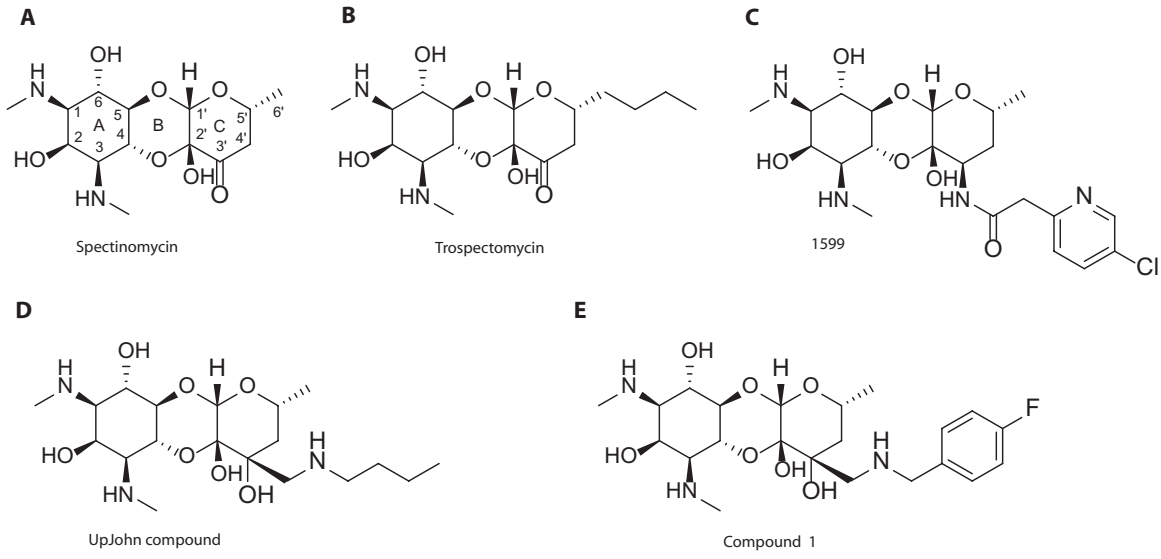


Fig. 1. Structure of spectinomycin and its analogs. (**A**) Spectinomycin, (**B**) trospectomycin, (**C**) spectinamide 1599, (**D**) a representative *N*-alkyl aminomethyl spectinomycin, and (**E**) compound 1, an example of *N*-benzyl aminomethyl spectinomycin explored in this study.

substituted aryl (principally 2-pyridyl) groups to the spectinomycin 3′ position of the exterior-facing C-ring via an acetamide linker. This modification restricts efflux of the spectinamides by Rv1258c, the pump that provides *Mycobacterium tuberculosis* with intrinsic resistance to the parent drug spectinomycin, which is overexpressed in multidrug-resistant isolates (*17*). Given this success, we were interested in exploring whether any other spectinomycin analogs could be modified to improve antibacterial activity against other drug-resistant pathogens.

After a comprehensive literature review of prior spectinomycin modifications, we were drawn to further explore the 3′-aminomethyl-3′-hydroxy spectinomycin (amSPC) scaffold that was identified by UpJohn Company in the 1980s (*18*) as a backup series to their Trospectomycin program. In these early studies, substitutions to the 3'-aminomethyl position focused on simple alkyl substitutions (*19*), which produced the lead shown in Fig. 1D with some improved antibacterial activity. Notably, the UpJohn studies were performed without the benefit of ribosomal structure binding information. In the course of the development of our antitubercular spectinamides, we had mapped and modeled the binding capacity of a previously unknown and conserved ribosomal aromatic binding pocket adjacent to the helix 34 spectinomycin site. Our modeling experiments demonstrated that this pocket was also accessible with *N*-benzyl substitutions to the amino methyl side chain of the amSPCs. This collective knowledge was applied to modify the 3'-aminomethyl-3'-hydroxy spectinomycin core, generating the *N*-benzyl aminomethyl spectinomycins with favorable pharmacological properties and improved antibacterial activity for the treatment of drug-resistant bacterial infections.

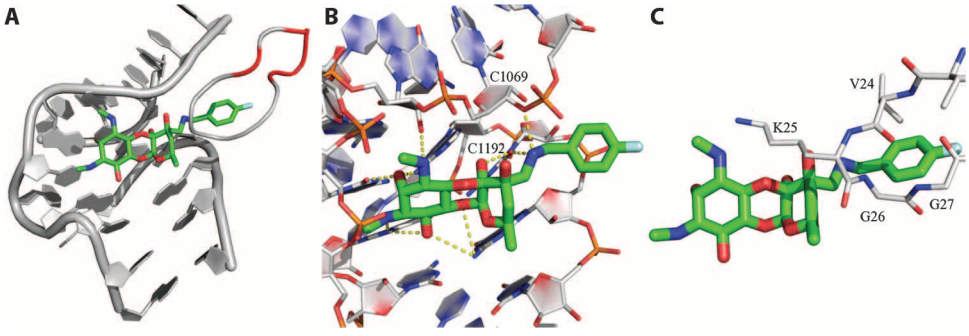


Fig. 2. Compound 1 modeled into the bacterial ribosome of *S. pneumoniae*, which shows the aryl side chain positioned in a side pocket adjacent to RpsE loop. (**A**) Structural variances between the *E. coli* and *S. pneumoniae* are highlighted in red within the loop of ribosomal protein RpsE, which contacts helix 34 of the 30*S* ribosomal spectinomycin binding site. (**B**) A magnified view of compound 1's predicted interaction with the *S. pneumoniae* ribosome. Hydrogen bonds are highlighted in yellow dashed lines. (**C**) Compound 1's predicted positioning within the *S. pneumoniae* RpsE loop.

RESULTS

Computational modeling suggests *N*-benzyl–substituted amSPCs should have broad spectrum inhibition of bacterial ribosomes

To guide our synthesis of the *N*-benzyl aminomethyl spectinomycins (amSPCs), we took advantage of structure-based design methods that we previously applied to the spectinamide series (*17*). Using the crystal structure of spectinomycin bound to the *Escherichia coli* ribosome, we built a homology model of the *S. pneumoniae* ribosome as a representative Gram-positive spectinomycin binding site (Fig. 2) (*7*). Within a 15-Å sphere centered at the binding site, the nucleic acids were highly conserved with just a single variance (A1081G, *E. coli* numbering) noted. The structure of the RpsE protein loop displayed more species-specific heterogeneity, especially in regions more distal from the spectinomycin binding site (Fig. 2A and fig. S1). The four RpsE protein loop residues closest to the spectinomycin binding site (V24, K25, G26, and G27,

S. pneumoniae numbering), however, were fully conserved across all bacterial species investigated in this study (fig. S1). The sequences of mitochondrial ribosome RpsE from mammalian species (including human) were included in this alignment and noticeably distinct from bacterial RpsE sequences at several residues including K22M, V24A, and G26E (*S. pneumoniae* numbering, fig. S1). Docking and short molecular dynamics simulations were performed to investigate the putative binding of amSPC compounds. The *E. coli* and *S. pneumoniae* ribosome models were used as representative Gram-negative and Gram-positive spectinomycin binding sites, respectively. These in silico analyses suggested that a modified 3' benzyl side chain could fit well into an extended binding pocket composed of both nucleic acids and the RpsE protein loop. Modeling predicted that the NH_2^+ group in the 3′ methylene side chain can form a hydrogen bond with the cytosine carbonyl of C1069 as well as the keto group in C1192 to constrain the newly introduced side chain of amSPCs (Fig. 2B). Only K25 (*S. pneumoniae* numbering) of the RpsE loop residues forms a direct ribosome contact, making a stabilizing salt bridge with helix 34. The other three residues form a pocket to accommodate the 3′ benzyl side chain of amSPCs, similar to what we had predicted for the aryl binding site of the spectinamide series (Fig. 2C). These protein residues (V24, G26, and G27) appeared to stabilize the binding of the amSPC compounds via mostly lipophilic interactions. No major interactions or conformational changes were observed in the molecular dynamics simulation upon changing the corresponding outer RpsE loop residues in *E. coli* to *S. pneumoniae* sequence (S21T, T23V, I329R, F30L) because these residues were located too far away from the *N*-benzyl amSPC side chain to make contact. Together, our analysis of the spectinomycin binding site and the helix 34/RpsE interface indicated that *N*-benzyl–substituted amSPC should inhibit ribosomes across a broad spectrum of bacterial pathogens.

N-benzyl aminomethyl spectinomycins were synthesized based on in silico modeling

On the basis of our in silico modeling results, targeted 3′ *R*-isomer amSPCs (compounds 1 to 4) were designed and synthesized from spectinomycin in a five-step sequence according to the procedure of Thomas, using optimized protocols for the reductive amination and benzyloxy carbamate deprotection steps (method S1 and scheme S1) (*20*, *21*). The corresponding 3′ *S*-isomer controls (compounds 5 and 6) were generated by an identical approach (scheme S2) with exception of the key hydrocyanation step, which was performed using acetone cyanohydrin in methanol under basic conditions to obtain the 3′ *S*-isomers. To date, we have synthesized a total of 132 variously substituted aminospectinomycins, including 20 *N*-benzyl aminomethyl spectinomycins. This report describes a subset of the *N*-benzyl aminomethyl spectinomycins, selected for the best clinical potential as judged by their balance of improved antibacterial activity, favorable pharmacokinetic properties, and in vivo efficacy.

amSPCs inhibit bacterial but not mammalian protein synthesis

The substituted amSPCs were tested for inhibition of bacterial protein synthesis using in vitro bacterial translation assays (Table 1) (*22*). In agreement with in silico modeling, *N*-benzyl–substituted amSPCs (typified by the unsubstituted core molecule compound 2) were potent inhibitors of bacterial protein translation with IC_{50} (50% inhibitory concentration) values similar to spectinomycin (Table 1). Halide substitutions to the para position of the phenyl ring were explored via the addition of fluorine (compound 1), trifluoromethoxy (compound 3), and chlorine (compound 4) because similar substitution patterns were found to be favorable in the spectinamide series and to also block a site of potential oxidative metabolism. Introduction of the halide substituents were well tolerated, with the chloro substitution (compound 4) producing the most potent protein translation inhibitor. The corresponding *S*-isomers of compounds 1 (compound 5) and 3 (compound 6) were synthesized (scheme S2) and tested. As predicted from modeling experiments, the *S*-isomers had only weak inhibitory activity in protein translation assays compared to their corresponding *R*-isomers (Table 1). Inhibition of protein synthesis by the amSPCs was restricted to bacterial ribosomes, as they produced no inhibition of mammalian ribosomes (table S2). This reflects the inherent differences in 16*S* ribosomal RNA (rRNA) helix 34 and RpsE between bacterial and mammalian ribosomes. Both cytosolic and mitochondrial mammalian ribosomes have considerable nucleotide polymorphisms in the 16*S* rRNA helix 34 when compared to the conserved bacterial sequence, which precludes binding of the spectinomycin core to mammalian ribosomes (*17*). The replacement of the more bulky, negatively charged glutamate at RpsE loop residue 26 in both mammalian ribosomes with glycine in bacteria affords an additional opportunity for amSPCs to avoid nonspecific activity toward host cells.

amSPCs are noncytotoxic and avoid human targets and metabolizing enzymes

Mammalian cytotoxicity analysis was performed in vitro using Vero cells and hemolysis assays. The amSPCs were noncytotoxic against mammalian cells and nonhemolytic against erythrocytes at concentrations >100 μg/ml. Further, in vitro testing for compound 1 revealed no significant interaction with a panel of 68 primary human molecular targets and 5 cytochrome P450 drug-metabolizing enzymes, suggesting minimal potential for adverse reactions and drug-drug interactions in vivo (table S3).

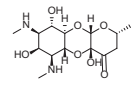
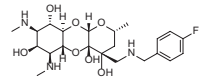
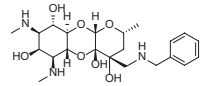
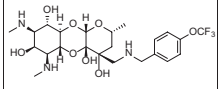
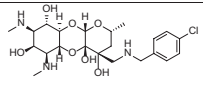
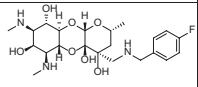
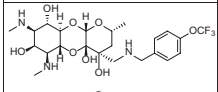
Gram-positive pathogens are more susceptible to amSPCs than spectinomycin

The ability of the amSPCs to reach their intracellular ribosomal target and inhibit bacterial growth was evaluated against a panel of 19 clinically important Gram-positive and Gram-negative pathogens (Table 1 and table S1). The minimum inhibitory concentration (MIC) of the amSPCs improved against Gram-positive pathogens when compared to that of spectinomycin. The greatest potency was seen for *Streptococcus* species with MICs for ribosomally active compounds ranging from 0.8 to 12 μg/ml. Substantial improvement in activity was also seen for *Enterococcus faecalis*, where addition of the unsubstituted aminomethyl benzyl group decreased the MIC from 100 to 25 μg/ml (compound 2) and introduction of the chloro substituent into the aryl ring further decreased the MIC to 6 μg/ml (compound 4). Against *Listeria monocytogenes*, a cause of serious foodborne illness, the MIC was reduced from 50 μg/ml for spectinomycin to 6 μg/ml (Table 1) for compounds 1 and 4. Like spectinomycin, amSPCs had only moderate or no activity against the Enterobacteriaceae, *Pseudomonas*, and *Acinetobacter* Gram-negative bacteria (table S1). As expected, non–ribosome-binding *S*-isomers were inactive against species sensitive to corresponding ribosome-binding *R*-isomers, further indicating that the series remains on target, exerting antibacterial activity by sustained ribosomal inhibition.

amSPCs lack cross-resistance to existing antibiotic classes

S. pneumoniae is an important respiratory pathogen causing pneumonia, otitis media, and meningitis, for which drug resistance is an increasing

Table 1. Ribosomal, cytotoxic, and Gram-positive activity of amSPC analogs. *Spn*, *Streptococcus pneumoniae* (R6); *Spy*, *Streptococcus pyogenes* (ATCC 700294); MSSA, *Staphylococcus aureus* (ATCC 29213); MRSA, *Staphylococcus aureus* (NRS70); *Ef*, *Enterococcus faecalis* (ATCC 33186); *Bs*, *Bacillus subtilis* (ATCC 23857); *Lm*, *Listeria monocytogenes* (NS512). Ribo., ribosomal.

Structure	IC_{50} μg/ml		MIC μg/ml*						
	Ribo.†	Vero‡	*Spn*	*Spy*	MSSA	MRSA	*Ef*	*Bs*	*Lm*
	0.39	>100	12.5	25	100	>200	100	12.5	50
 1 (*R*)	0.74	>100	1.6 – 3.1	1.6	25 – 50	50	12.5 – 25	3.1	6.3
 2 (*R*)	0.87	>100	6.3 – 12.5	3.1	50	50 – 100	25	3.1	25
 3 (*R*)	1.15	>100	3.1	1.6	100	100 – 200	25 – 50	100	12.5 – 25
 4 (*R*)	0.30	>100	1.6	0.8 – 1.6	25 – 50	12.5 – 25	6.3 – 12.5	3.1	6.3
 5 (*S*)	16.6	>100	200 – >200	100	>200	>200	>200	200 – >200	>200
 6 (*S*)	20.1	>100	>200	>200	200 – >200	200 – >200	>200	100 – 200	–

*MIC results presented are the range of two biologically independent experiments. Dashes indicate where values have not been determined. †IC_{50} values were determined using ribosomes purified from *Mycobacterium smegmatis*. ‡Cytotoxicity IC_{50} was determined in vitro using mammalian cells (Vero), and the average of two separate experiments is presented, with SEM provided in parentheses.

problem (*1*). As the amSPCs were most active against pneumococci, they were further tested against a panel of 15 *S. pneumoniae* clinical isolates. All strains tested remained susceptible to amSPCs, including isolates resistant to penicillin G, streptomycin, clindamycin, macrolides, and cefotaxime (Table 2). The amSPCs tested were active against a strain (BAA-1407) harboring both of the macrolide resistance genes *mef*E (commonly distributed in Europe and Asia) and *erm*B (most commonly found in the North America) (*23*). amSPCs were also active against an isolate (strain 8249) with resistance to cephalosporins by high-level expression of an altered penicillin binding protein. To further test for cross-resistance potential, *S. pneumoniae* mutants resistant to compound 1 were generated in vitro (arising at a frequency of 5.7×10^{-11} to 2.9×10^{-10}). Fifteen resistant clones were isolated and exhibited high-level resistance to compound 1 (MIC >200 μg/ml). All clones were tested for cross-resistance and remained sensitive to existing classes of antibiotics (table S4). Despite high-level resistance to compound 1, the clones remained weakly susceptible to the parent spectinomycin. To rationalize this result, mutant genomes were sequenced, and whereas no mutations were identified in the RNA helix 34, a mutation in RpsE (G27R) was identified (fig. S2). In silico docking of spectinomycin and compound 1 predicted this mutation to exert a greater impact on the binding of compound 1 as compared to spectinomycin (fig. S3). This is in agreement with the drug sensitivity observed, and further indicated that the antibacterial activity of amSPCs is a consequence of ribosomal inhibition. The susceptibility of drug-resistant pneumococci to amSPCs and the lack of cross-resistance with compound 1 in in vitro mutants most likely reflects the fact that the ribosomal binding site for spectinomycin and its derivatives is distinct from that of other antibiotics and indicates the potential for treatment of drug-resistant pneumococcal infections.

Common bacterial respiratory pathogens are susceptible to amSPCs

Treatment of respiratory tract infections typically involves a cephalosporin, a macrolide, or the combination of both classes of these antibiotics, but macrolide resistance threatens to undermine the effective

Table 2. Antipneumococcal activity of select amSPCs against drug-sensitive and drug-resistant isolates. MIC testing for amSPC activity against a panel of *S. pneumoniae* isolates.

Treatment	MIC (μg/ml)																	
	Drug-sensitive					Drug-resistant												
	R6	T4X	D39X	BHN97x	A66.1x	OVA2	BAA-1407	Daw7	Daw8	Daw9	Daw62	Daw64	Daw19	Daw26	Daw27	Daw47	8249	ATCC700904
SPC	13	13	13	6	6	13	13	25	25	13	25	25	25	50	25	25	25	25
1	3	3	2	2	2	3	6	3	3	3	3	3	6	6	6	6	3	6
2	6	6	3	3	3	6	13	6	6	6	6	6	13	13	13	13	6	13
3	3	1	—	—	—	6	6	3	2	2	2	3	6	6	3	3	2	13
Penicillin G	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2	2	3	3	2	3	2	3	3	3	3	>2	>2
Cefotaxime	—	—	—	—	—	—	—	—	—	—	—	—	1	3	0.4	3	3	0.2
Erythromycin	≤0.2	—	—	—	—	1	50	≤0.2	≤0.2	2	≤0.2	<0.2	1	0.1	1	0.03	>2	2
Streptomycin	13	6	13	13	13	13	50	25	25	>200	25	25	—	—	—	—	>200	>200

treatment of atypical and severe respiratory diseases. Because drug-resistant *S. pneumoniae* remained sensitive to amSPCs, we next sought to determine if the series was active against other common bacterial respiratory pathogens. Potency was improved against *Haemophilus influenzae*, where the zone of inhibition increased from 12 mm for spectinomycin to 19 mm for compounds 1 and 3 (Table 3), with the larger zone of inhibition indicating increased antibacterial activity. *Moraxella catarrhalis* is a fastidious pathogen involved in upper respiratory tract infections and is susceptible to spectinomycin (MIC of 6 μg/ml). The amSPCs maintained activity of the parent spectinomycin toward this Gram-negative pathogen, with compound 1 active at a concentration of 3 μg/ml (table S1). *Burkholderia cepacia* causes pulmonary disease in cystic fibrosis patients and is notoriously difficult to treat owing to its resistance to common antibiotics. We were able to improve potency from 25 to 6 μg/ml against *B. cepacia* (table S1). The improvement in activity of the amSPCs over spectinomycin was most pronounced against *Legionella pneumophila* (Table 2). This species has a chromosomally encoded aminoglycoside-modifying enzyme that inactivates spectinomycin to provide intrinsic resistance (*24*). As anticipated, we found spectinomycin completely inactive against *L. pneumophila*. The amSPCs, however, were potent *L. pneumophila* inhibitors that produced zones of inhibition ranging from 20 to 40 mm (Table 2 and fig. S4), suggesting that they overcome intrinsic resistance. Together, these data demonstrate that amSPCs have superior activity to spectinomycin against common bacterial respiratory pathogens.

N. gonorrhoeae and *Chlamydia trachomatis* are sensitive to amSPCs

Historically, the only clinically approved indication for spectinomycin (Trobicin) was for the treatment of complicated gonorrheal infections, until production for the U.S. market was discontinued in 2005. However, isolates resistant to all available treatments including the cephalosporin ceftriaxone have arisen recently and now pose a threat to public health worldwide (*25*, *26*). This underscores the need for new treatments for this sexually transmitted disease. The amSPCs were tested for activity against *N. gonorrhoeae* and closely related *Neisseria meningitidis* (Table 3). Compound 4 demonstrated the best activity among the compounds in this set, producing a zone of inhibition of 20 to 22 mm,

Table 3. Zone of inhibition testing against fastidious Gram-negative pathogens. Zone of inhibition testing for amSPCs against fastidious pathogens. Results presented are the range of two biologically independent experiments. Dashes indicate where values were not determined. *Ng*, *Neisseria gonorrhoeae* (ATCC 49226); *Nm*, *Neisseria meningitidis* (ATCC 13077); *Hi*, *Haemophilus influenzae* (ATCC 49247); *Lp*, *Legionella pneumophila* (ATCC 33153).

Compound	*Ng*	*Nm*	*Hi*	*Lp*
Spectinomycin	10–11.5	13–15	11–12.5	0
1	16–19	19–20	17–19	32–33
2	15.5–16	16–21.5	15.5	40
3	16–20	18–19	16	21
4	20–22	16–18	15–16	21–22
5 (*S*)	0	0	0	0
6 (*S*)	8.2–8.8	—	—	8

double the 10- to 11-mm zones produced by spectinomycin (Fig. 3). Because the amSPCs showed improved activity against *N. gonorrhoeae*, we also tested their potency against *Chlamydia trachomatis*, an intracellular pathogen that commonly co-infects persons with *N. gonorrhoeae* (Fig. 3 and fig. S5). Spectinomycin concentrations of up to 100 μg/ml failed to reduce intracellular inclusions in *C. trachomatis*–infected monolayers; however, compounds 1 and 4 at a concentration of 12 μg/ml reduced intracellular bacterial loads by more than 50%. HeLa cell monolayers were not disrupted even at 100 μg/ml, the highest concentration of compounds tested. This is in agreement with cytotoxicity testing and indicates that reduction in intracellular *C. trachomatis* produced by compounds 1 and 4 arises from their antichlamydial activity. Activity against *Chlamydia* species is notable, given its close phylogeny to *Chlamydia pneumoniae*, a respiratory pathogen that commonly causes pneumonia. The dual activity of amSPCs against both *N. gonorrhoeae* and *C. trachomatis* is consistent with published data for trospectomycin, a 5′-spectinomycin derivative that is efficacious in humans for treatment of both gonorrhea and chlamydia infections (*15*, *27*, *28*).

N-Benzyl aminomethyl spectinomycins have a unique spectrum of activity

The spectrum of antibacterial activity of our *N*-benzyl–substituted amSPCs appeared to differ from that reported for the *N*-alkyl aminomethyl spectinomycins (*19*). To further assess the differences in activity between these closely related yet chemically distinct spectinomycins, we resynthesized two of the most potent alkyl analogs previously reported by UpJohn Company as having improved antibacterial activity. These alkyl-substituted analogs showed no improvement in potency over spectinomycin toward the bacterial therapeutic targets discussed in this article, with the exception of *L. pneumophila* (table S5). The antibacterial spectrum of the amSPCs is also notably different from that of the previously reported spectinamides (*17*). The spectinamides are narrow-spectrum inhibitors with a log greater activity against *M. tuberculosis* than spectinomycin and have little to no improvement in activity over pathogens outside the *M. tuberculosis* complex. Conversely, the amSPCs have a wider spectrum of antibacterial activity but lack potency against *M. tuberculosis* (table S1). These results indicate that *N*-benzyl aminomethyl spectinomycins have a spectrum of activity unique from those of previously reported spectinamides and alkyl amSPCs.

amSPCs have favorable in vitro and in vivo pharmacokinetic parameters

Compounds 1, 3, and 4 were selected for pharmacokinetic profiling based on their antibacterial properties to determine if they were suitable for in vivo efficacy trials (Table 4) (*29*). All three compounds exhibited increased plasma protein binding compared to spectinomycin but still in a relatively low range (43 to 63%), thereby providing a large fraction of the compound in the unbound, pharmacologically active form. In vitro metabolic stability assessments in rat hepatic microsomal preparations demonstrated that all compounds had high metabolic stability, either similar to (compound 1) or better than (compounds 3 and 4) spectinomycin.

The chemical stability of compound 1 was examined in comparison to spectinomycin at pH 2, 7, and 9 (method S2, table S6, and fig. S6) to determine if replacement of the chemically reactive keto group of spectinomycin (*30*) with the chemically stable 3′ aminomethyl functionality improved the stability of the series. As anticipated by their inability to undergo the actinospectose rearrangement, much improved chemical stability of the amSPCs was observed at pH 7 and 9 as compared to spectinomycin. The percent of parental spectinomycin continuously declined over time at pH 7 and 9, whereas compound 1 was not degraded even when incubation time was extended to four full days. This is in agreement with our previous spectinamide series (*17*) and highlights the benefit of eliminating spectinomycin's reactive keto group to produce the amSPC series.

Because in vitro testing indicated that amSPCs maintain spectinomycin's favorable safety profile but were less reactive, in vivo pharmacokinetic characterization was performed. The pharmacokinetic profile of compounds 1, 3, and 4 was examined in rats via intravenous administration and compared to previous data we had derived for spectinomycin under identical conditions (Table 4) (*31*). In these experiments, amSPCs were well tolerated in the animals at a dose of 10 mg/kg with no adverse effects noted. All compounds showed a similar and predictable systemic exposure, with peak plasma concentrations of 19.7 to 21.5 μg/ml and an area under the curve of 17.4 to 19.4 μg hour/ml. The compounds exhibited biexponential plasma concentration-time profiles with a half-life of 1.12 to 1.99 hours at therapeutically relevant concentrations above the MIC, which is longer than the 0.75 hour observed for spectinomycin in rats. Because clearance was similar to spectinomycin for all compounds, the longer half-life observed for compounds 3 and 4 is the result of an increased volume of distribution compared to spectinomycin. In agreement with the high in vitro metabolic stability and similar to spectinomycin and other aminocyclitol antibiotics, renal excretion is the major elimination pathway for amSPCs, with 58 to 82% excreted unchanged in urine, except for compound 3 with only 22% renal excretion. These results suggest that compounds 1, 3, and 4 have many pharmacokinetic properties desirable for antibacterial drugs and were suitable for progression into in vivo efficacy trials.

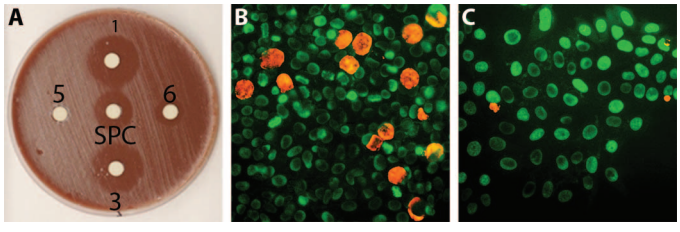


Fig. 3. Activity against *N. gonorrhoeae* and *C. trachomatis*. (**A**) Zone of inhibition testing for *N. gonorrhoeae*. A representative image of disc diffusion assays for *N. gonorrhoeae* is shown. Compound (40 μg) dissolved in dimethyl sulfoxide (DMSO) was applied to the disc. (**B** and **C**) Representative images of mCherry-expressing *C. trachomatis* (orange) infected monolayers (green) treated with 12 μg/ml (B) spectinomycin or (C) compound 1.

amSPCs rescue mice from a lethal pneumococcal infection

amSPCs 1, 3, and 4 were tested for antipneumococcal activity in mice infected with *S. pneumoniae* strain D39, which causes pneumonia and

Table 4. Pharmacokinetic parameters for select amSPCs. Parameters are expressed as mean. Values in parentheses indicate %CV. $t_{1/2}$, half-life for metabolic degradation; $T_{1/2}$, Pharmacokinetic half-life; Vd, volume of distribution; CL, clearance; f_e, fraction excreted unchanged in urine.

Compound	Protein binding	Microsomal stability	Intravenous pharmacokinetics (dose, 10 mg/kg)			
	%Bound	$T_{1/2}$ (hours)	$t_{1/2}$ (hours)*	Vd (liters/kg)	CL (liters hour^{-1} kg^{-1})	f_e
SPC†	13.0 (7.5)	6.43 (0.13)	0.75 (49.3)	0.76 (45.2)	0.60 (11.5)	0.55 (27.0)
1	43.0 (1.7)	6.80 (0.56)	1.12 (14.2)	0.64 (39.1)	0.56 (23.0)	0.82 (12.4)
3	62.6 (8.8)	28.8 (2.19)	1.99 (11.9)	1.13 (32.7)	0.53 (22.2)	0.22 (28.1)
4	57.6 (11.9)	23.1 (1.64)	1.74 (2.8)	1.45 (12.7)	0.58 (5.4)	0.58 (4.7)

*$t_{1/2}$ is based on decline of plasma concentration in the therapeutically relevant concentration range. †Spectinomycin values are from (*31*) and included for comparison.

bacteremia. Compounds were administered subcutaneously twice daily to infected mice 18 hours after intranasal challenge, a time when mice have developed both pneumonia and bacteremia (*32*). All mice receiving compound 1 or 4 at the lowest dose (5 mg/kg) survived, whereas all mice receiving vehicle and spectinomycin controls died or were humanely euthanized by 120 hours after challenge (Fig. 4, A and C). Compound 3 improved survival for three of five mice at 5 mg/kg, but was not as protective as compounds 1 and 4 (Fig. 4B). The bacterial burden in the blood 48 hours after challenge was reduced significantly ($P < 0.05$) in groups receiving compound 1 or 4 compared to the spectinomycin and vehicle controls (Fig. 4, D and F). Clearance of the infection was also evident in the bioluminescent images of mice at 72 hours after challenge, which showed systemic bacterial infection in both the vehicle and spectinomycin groups, whereas mice administered compounds 1 and 4 showed clearance of infection below detectable limits (Fig. 4, G to I).

To determine how the efficacy of compound 1 compared to ampicillin, an antibiotic used for *S. pneumoniae* infections, we tested the efficacy of matched doses of compound 1 and ampicillin (fig. S8). Mice administered either compound 1 or ampicillin (matched at 2.5 or 5 mg/kg) all survived the 9-day experiment, whereas four of the five mice administered the carrier died by day 5 after infection. Within 48 hours after infection, bacterial titers were reduced by more than 3 logs in the treatment groups and were barely above the limit of detection. Reduction of bacterial burden provided by compound 1 was equivalent to that of ampicillin at matched doses, indicating that under these conditions, compound 1 is as effective as ampicillin at protecting mice from pneumonia and bacteremia.

Compound 1 was tested for efficacy in a second mouse model using *S. pneumoniae* strain TIGR4, which causes severe bacteremia and meningitis (fig. S7). Mice were administered vehicle, spectinomycin (5 mg/kg), or compound 1 (5 mg/kg) beginning 18 hours after intranasal challenge with strain TIGR4. Mice receiving vehicle or spectinomycin did not survive beyond 96 hours of the initial bacterial challenge, whereas compound 1 rescued 80% of mice from this highly lethal infection. These results indicate that amSPCs mediate significantly greater protection than spectinomycin, preventing the progression of fatal pneumococcal pneumonia, meningitis, and sepsis.

DISCUSSION

Here, we apply structure-based design to generate a chemically distinct spectinomycin series that maintains ribosomal target affinity while increasing antibacterial potency. Two groups of clinically relevant pathogens were identified as targets for potential treatment with the new analogs: drug-resistant sexually transmitted and drug-resistant respiratory bacterial pathogens, including several highlighted in the U.S. Centers for Disease Control and Prevention (CDC) 2013 report on antibiotic resistance threats (*1*). This work successfully moved the antibacterial activity of the series into a therapeutic range for these agents while also improving pharmacokinetic properties. Compounds in this series have a mechanism of action distinct from that of other antibiotics, including other protein synthesis inhibitors, are highly selective toward bacterial ribosomes, and have negligible cytotoxicity. We verified the consequence of these improvements, demonstrating in vivo efficacy against lethal pneumococcal pulmonary challenge. Thus, we have expanded the potential of spectinomycins to treat drug-resistant infections for which very few treatment options are available.

The amSPC-target interaction at the ribosome was tolerant of the benzyl substitutions introduced, provided the 3′-(*R*)-aminomethyl stereochemistry of the substitution was maintained. This presented the opportunity to introduce modifications that improved antibacterial activity and stability without decreasing ribosomal potency. Improvement in activity against whole cells was not related to increased ribosomal affinity, suggesting that the molecular basis for their superior MIC is extra-ribosomal. Studies involving the

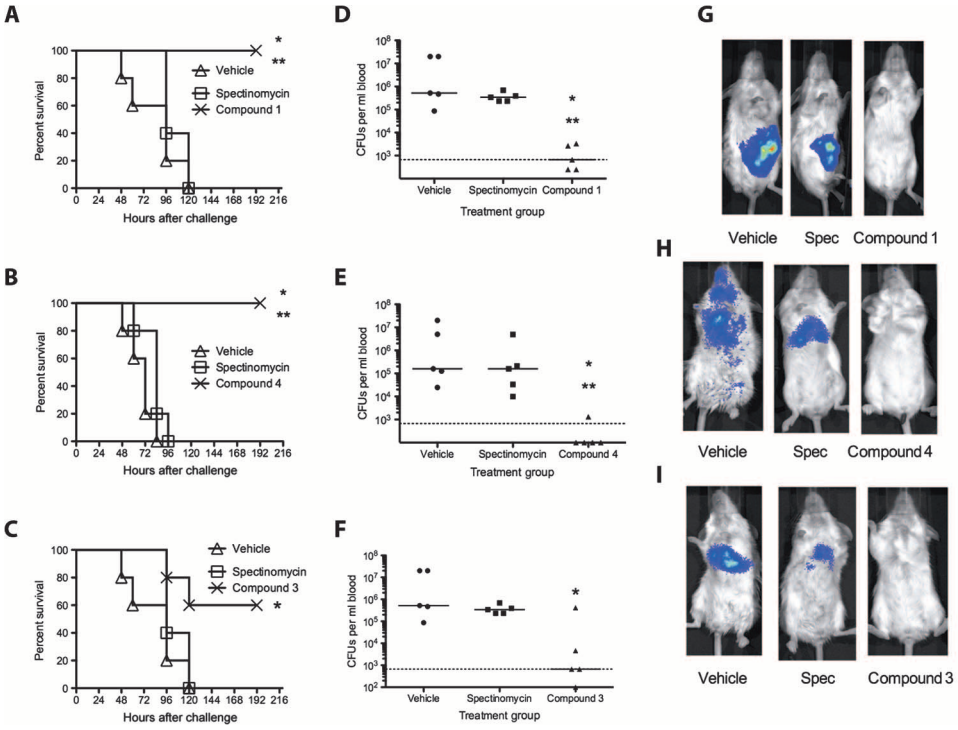


Fig. 4. amSPCs mediate more effective protection than spectinomycin from invasive pneumococcal challenge. A dose of 5 mg/kg was administered twice daily beginning 18 hours after challenge with *S. pneumoniae* D39x. (**A** to **C**) Overall survival of mice receiving vehicle control or the indicated compounds ($n = 5$ mice per group). (**D** to **F**) Bacterial burden in the blood at 48 hours after challenge. (**G** to **I**) Representative bioluminescent images of mice at 72 hours after challenge. Statistical significance was determined using log-rank test (Mantel-Cox) for survival data and Mann-Whitney test for bacterial burden data. *$P < 0.05$ when compared to vehicle control group. **$P < 0.05$ when compared to spectinomycin control. *P* values were as follows: (A) *$P = 0.0041$, **$P = 0.0023$; (B) *$P = 0.0021$, **$P = 0.0020$; (C) *$P = 0.023$; (D) *$P = 0.0117$; (E) *$P = 0.0097$; (F) *$P = 0.0208$.

antitubercular spectinamide series demonstrated that their improved activity resulted from greater accumulation of the active compound within the bacteria due to lack of efflux by Rv1258c (*17*). It is likely that the amSPCs also have greater accumulation within target bacteria and that avoidance of species-specific drug-efflux mechanisms is also key to the activity of this series.

The CDC recently classified *N. gonorrhoeae* as an urgent threat, the highest priority category for a bacterial infection in the United States. It is notable that for the treatment of the multidrug-resistant *N. gonorrhoeae* H041 strain, which is causing much clinical concern, spectinomycin is one of the few therapeutic choices remaining (*33*). In the current study, the spectinomycin analogs synthesized had better activity than spectinomycin against both *N. gonorrhoeae* and *C. trachomatis*. This improvement in bioactivity when combined with a longer pharmacokinetic half-life and improved chemical stability suggests that treatment with amSPC could be achieved at a lower dose than the 2 g required currently for therapy with spectinomycin for gonorrhea. Unfortunately, preclinical testing of antigonorrheals is hindered by poor colonization of lower vertebrates (including rodents) and the high costs and ethical issues of using nonhuman primate models for gonorrhea infection (*34*). Thus, our preclinical development of the amSPCs for this indication will likely use a pharmacokinetic/pharmacodynamic guided approach, utilizing animal efficacy data from other bacterial species to project human dosing requirements. This strategy has been successfully used by AstraZeneca for the development of novel antigonorrheal candidate AZD0914, which has been given Fast Track status by the U.S. Food and Drug Administration (*35*).

Improved activity of the amSPCs was seen in vitro against the most common respiratory pathogens and causes of bacterial otitis media including *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *L. pneumophila*. amSPCs were also potent inhibitors of *S. pyogenes*, which causes pharyngitis and necrotizing fasciitis and can be challenging to treat in patients allergic to β-lactams because of increasing clindamycin and macrolide resistance (*36*–*38*). Because *S. pneumoniae* is a predominant cause of childhood upper respiratory tract infections and treatment is compromised by resistance to macrolides and β-lactams, we focused our in vivo efforts on this pathogen. We demonstrate that amSPCs have potent in vivo activity capable of clearing high-burden, lethal *S. pneumoniae* infections at low doses. *mef* E efflux was our greatest concern with respect to preexisting cross-resistance mechanisms that might influence amSPC potency against streptococci, but this efflux system had little effect on amSPC activity. The lack of cross-resistance seen in clinical isolates and in evaluation of spontaneous mutants strongly suggests that the amSPCs have potential to successfully treat infections resistant to existing therapeutics.

The primary limitations of the series are low oral bioavailability of amSPCs, despite the improved chemical and metabolic stability, and lack of adequate *S. aureus* efficacy. We recently demonstrated success in nebulized delivery of spectinamide derivatives in the treatment of the respiratory pathogen *M. tuberculosis* (*17*), a route of delivery that produces substantial systemic availability of the drug. This suggests that aerosol delivery may be appropriate for delivery of amSPCs for treatment of respiratory infections, as the physicochemical properties of both series are very similar. Although activity against *S. aureus* is desirable, this bacteria is a less common cause of respiratory tract infections and is a minor cause of community-acquired bacterial pneumonia for which there are other therapeutic options.

We have previously demonstrated that spectinomycin selectivity toward bacterial ribosomes and avoidance of human mitochondrial ribosomes, despite the bacterial origin of this organelle, is explained by sequence variations within RNA and the associated RpsE protein portion of the spectinomycin binding site. Among the bacterial species included in our analysis, RpsE V24 and G26 residues are 100% conserved and predicted to stabilize the binding of amSPCs within the bacterial ribosome. Conversely, the corresponding positions in human mitochondrial RpsE are distinct (A233 and E235) and predicted to occlude amSPCs from binding. In addition, we demonstrate that amSPCs do not inhibit mammalian protein synthesis even at very high concentrations. No acute toxicity was noted for the amSPCs at the dose ranges studied, as anticipated from the impressive safety profile of the parent spectinomycin. However, further toxicity studies remain a critical component of the future preclinical development for amSPCs.

The pharmacokinetic profile of the amSPCs is very similar to spectinomycin, especially for compound 1. This is desirable from a drug development point of view because high hydrophilicity and solubility result in limited protein binding, good tissue access, and limited drug metabolism. The chemical stability of amSPCs, however, is much improved because the chemically reactive keto group within spectinomycin has been removed. The amSPCs are highly efficacious, clearing lethal *S. pneumoniae* pulmonary infections in mice at low doses, performing better in vivo than in vitro susceptibility testing alone would suggest. This is clearly in part due to their good pharmacokinetic properties, as we have observed with the spectinamide series.

Our recent studies have reevaluated the potential for synthetic modification of the spectinomycin core. Using new ribosome binding information and prior chemical literature from the 1970s and 1980s, we produced the antitubercular spectinamides and the second-generation amSPCs reported herein. These studies each generated a chemically distinct series of spectinomycins with nonoverlapping disease indications. The spectinamides have excellent activity specific to the *M. tuberculosis* complex, whereas the *N*-benzyl–substituted amSPCs have robust activity against resistant respiratory tract and sexually transmitted bacterial pathogens. These studies highlight the potential of modifying the 3′ position of the exterior-facing C-ring in spectinomycin to modulate the antibacterial spectrum of activity while maintaining ribosomal inhibitory potency. This strategy facilitates our ability to generate new therapeutic agents suitable to treat drug-resistant bacterial infections.

MATERIALS AND METHODS

Computational studies

For computation and bioinformatics analyses across species, *S. pneumoniae* numbering was used to indicate specific nucleotides and amino acid residues unless otherwise indicated. The cocrystal structure of spectinomycin bound to the 30*S* ribosome from *E. coli* (Protein Data Bank ID: 2QOU) was used for construction of a homology model of the spectinomycin binding site from S. *pneumoniae*, using the same approach that we applied to the *M. tuberculosis* ribosome (*17*). Primary sequence variances within a 15-Å truncated sphere centered at the binding site A1081G (S22T, T24V, I30R, F31L) were identified. Mutations were then introduced by the "Mutate Residues" script implemented in Maestro (Schrodinger). Both *E. coli* and *S. pneumoniae* spectinomycin binding site models were prepared for docking using Protein Preparation Wizard (Schrodinger). Compounds were docked using Glide

SP to each receptor structure (*39*). The top-scoring docking conformations were used to initiate a 5-ns molecular dynamics simulations, which was performed as described (*17*).

Ribosomal inhibition assays

S30 extracts or purified ribosomes were used in translation reactions of luciferase mRNA as described (*40*). Firefly (F-luc) mRNA was produced in vitro using T7 RNA polymerase (Fermentas). A typical translation reaction mixture with a total volume of 30 μl contained 0.25 μM 70*S* ribosomes, 4 μg of F-luc mRNA, 40% (v/v) S100 extract, 200 μM amino acid mixture, 24 U of RiboLock (Fermentas), transfer RNAs (tRNAs; 0.4 mg/ml), and 12 μl of commercial S30 Premix without amino acids (Promega). After addition of serially diluted spectinomycin derivatives, the reaction mixture was incubated at 37°C for 35 min, the reaction was stopped on ice, and the reaction mixture assayed for F-luc luciferase activities using the Luciferase Reporter Assay System (Promega). Luminescence was measured using a luminometer (FLx800, Bio-Tek Instruments). Drug-mediated inhibition of protein synthesis is expressed as IC_{50}, that is, the drug concentration that results in 50% inhibition of luciferase synthesis. When purified bacterial ribosomes were used, these were isolated from *M. smegmatis* to permit comparison with our previously published data for spectinomycin derivatives. We have previously shown a correlation of the data for inhibition of *E. coli* and *M. smegmatis* and noted that the expression the 16*S* rRNA from a single gene in *M. smegmatis* (as opposed to multiple gene copies in other prokaryotes including *E. coli*) affords the opportunity to dissect ribosomal interactions via mutagenesis.

Bacterial growth

B. subtilis (ATCC 23857), *E. faecalis* (ATCC 33186), *S. aureus* (ATCC 29213), MRSA *S. aureus* (NRS70), *Acinetobacter baumannii* (ATCC 19606), *E. coli* (strain K12), *Klebsiella pneumoniae* (ATCC 33495), *Pseudomonas aeruginosa* (PA01), *Stenotrophomonas maltophilia* (ATCC 13637), and *B. cepacia* (ATCC 25416) were maintained in Mueller Hinton broth or on agar plates. *S. pneumoniae* (strain R6) and *S. pyogenes* (ATCC 700294) were cultured in Mueller Hinton in the presence of 10% (v/v, final) defibrinated, lysed horse blood. *L. monocytogenes* (NS512) and *M. catarrhalis* (ATCC 25238) were cultured in brain-heart infusion (BHI). *N. gonorrhoeae* (ATCC 49226), *N. meningitidis* (ATCC 13077), and *H. influenzae* (ATCC 49247) were maintained on GC agar supplemented with 2% (v/v) IsoVitaleX and 1% (w/v) hemoglobin. *L. pneumophila* (ATCC 33153) was maintained on CYE supplemented with BCYE. Agar plates for *N. gonorrhoeae*, *N. meningitidis*, and *H. influenzae* were sealed in gas-permeable bags (StarPac polyethylene bags from Garner US Enterprises) and incubated at 37°C in the presence of 5% CO_2. *M. tuberculosis* (H37Rv) was cultured in Middlebrook 7H9 broth (Difco Laboratories) supplemented with 10% albumin-dextrose complex and 0.05% (v/v) Tween 80.

Liquid MIC testing

MICs were determined in appropriate media (indicated above) according to Clinical Laboratory Standards Institute (CLSI), using twofold serial dilutions in 96-well plates (Thermo Scientific) starting at a drug concentration of 200 μg/ml (*41*). Plates were incubated in ambient air at 37°C, and MICs were recorded after 16 to 20 hours of incubation, except for *M. tuberculosis* MICs, which were recorded after 7 days of incubation. MICs for standard laboratory strains were repeated at least twice, whereas MICs were performed once for panels of clinical isolates.

Disc diffusion assays

A 4-μl aliquot of stock solution (10 mg/ml) (in 100% DMSO) was added to 6-mm discs placed on bacteria-coated agar plates. Plates were sealed in CO_2-permeable bags and incubated at 37°C with 5% CO_2 overnight (*N. gonorrhoeae*, *N. meningitidis*, and *H. influenzae*) or for 48 hours (*L. pneumophila*). The ranges presented are zones of inhibition derived from two biologically independent experiments.

Hemolysis assays

Defibrinated sheep blood (Colorado Serum Company) was centrifuged gently at 350 relative centrifugal force (rcf) for 20 min. The two upper layers (buffy coat and plasma) were discarded, and erythrocytes were washed three times in sterile phosphate-buffered solution (PBS), pH 7.4. After the third wash, cells were diluted to a final concentration of 5% (v/v) in PBS. Twofold serial dilutions of test compound and positive control Triton X-100 were prepared in PBS in 96-well, round-bottomed plates. An equal volume (100 μl) of washed erythrocytes was added. Plates were incubated for 1.5 hours at 37°C, at which point plates were centrifuged and supernatant was transferred to an optically clear, flat, white-wall 96-well plate, taking care not to resuspend unlysed cells. The absorbance of supernatants was then read at an optical density (OD) of 540 nm. Percent hemolysis was calculated using the following formula: % lysis = (OD_{540} of sample − OD_{540} of blank)/(OD_{540} of positive control).

Cytotoxicity testing

Vero (kidney epithelial cells; ATCC CCL-81) monolayers were trypsinized and seeded at 5000 cells per well (10 to 15% confluence) in white-wall, flat-bottomed 96-well microwell plates (Corning), using enriched Dulbecco's modified Eagle's medium (DMEM/high glucose; Hyclone) containing 10% fetal bovine serum (FBS, ATCC-30-2020). Plates were incubated overnight at 37°C in the presence of 5% CO_2. Drug-free medium was then replaced with medium containing serial dilutions of test compound or DMSO carrier. After an additional 72 hours of incubation, viability was indirectly measured using the CellTiter-Glo Luminescent Cell Viability (Promega) assay. Assay plates were read at peak emission wavelength of 560 nm on an EnVision Multilabel Plate Reader (Perkin Elmer). The concentration of test compounds that inhibited growth by 50% (the IC_{50}) was computed using nonlinear regression–based fitting of inhibition curves using log[inhibitor] versus response-variable slope (four parameters)—symmetrical equation, in GraphPad Prism version 6 (GraphPad Software). For each experiment, compounds were tested in duplicate. IC_{50} values presented are the range of two biologically independent experiments.

Chlamydial culture and susceptibility testing

C. trachomatis serovar L2 (strain 434/Bu) was grown in HeLa 229 cells cultivated at 37°C with 5% CO_2 in high-glucose DMEM (Cellgro, Mediatech) supplemented with 10% heat-inactivated FBS. Elementary bodies were purified on density gradients of RenoCal-76 (Bracco Diagnostics) as described previously (*42*). *C. trachomatis* used for infection was transformed with p2TK-SW2 mCherry plasmid according to Agaisse and Derre (*43*). HeLa 229 cells were grown on coverslips in six-well plates containing DMEM with 10% FBS. The monolayers were pretreated with diethylaminoethyl-dextran (30 μg/ml) and infected at a multiplicity of infection of 0.5. Experimental compounds and reference antibiotic spectinomycin (originally dissolved at 100 mg/ml in 100% DMSO) were diluted directly in culture medium and added

at time of infection to achieve final test concentrations of 100, 50, 25, 12.5, 6.3, and 3.1 μg/ml. Infected monolayers were incubated at 37°C in an incubator supplied with 5% CO_2 for 48 hours, at which point cells were fixed for microscopy. HeLa cells were stained with Hoechst 33342 (0.2 μg/ml, in mounting medium). Microscopy slides were viewed on a Zeiss Axioplan 2 imaging fluorescence microscope equipped with a motorized stage. Levels of infection were estimated by counting the number of mCherry-positive inclusions per field (*44*). Images were collected using a AxioCamHR camera and Zeiss Axiovision 4.8 software. Images were processed with only linear adjustment of brightness and contrast.

In vitro pharmacokinetic studies and analysis

Plasma protein binding was determined by equilibrium dialysis at 37°C using the RED device (Thermo Scientific). Microsomal metabolic stability of aminomethyl spectinomycin compounds was assessed in pooled rat liver microsomal preparations (CellzDirect) by monitoring the disappearance of the parent compound over an incubation period of 90 min, as described (*45*). Drug concentrations were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay, and calculations were as described (*31*).

In vivo pharmacokinetic studies

Concentration-time profiles for pharmacokinetic analysis were determined for compounds 1, 3, and 4 after intravenous administration by serial sampling in groups of five rats. Catheterized male Sprague-Dawley rats (femoral vein for drug administration and jugular vein for blood sample collection) weighing about 200 to 225 g were obtained from Harlan Biosciences. Animals were kept on a 12-hour light/dark cycle with access to food and water ad libitum. Animal studies were conducted according to the Animal Welfare Act and the *Public Health Service Policy on Humane Care and Use of Laboratory Animals*. The study protocol was approved by the institutional animal care and use committee of the University of Tennessee Health Science Center. Compounds 1, 3, and 4 were dissolved in PlasmaLyte and administered intravenously to a group of five rats (n = 5) at a dose of 10 mg/kg. Serial blood samples (about 250 μl) were collected at 0 (pre-dose), 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, and 48.0 hours after dose. Plasma was separated immediately by centrifugation (10,000*g* for 5 min at 4°C) and stored at −80°C until analysis. Urine samples were collected at an interval of 0 to 6, 6 to 12, 12 to 24, 24 to 36, and 36 to 48 hours after dose and stored at −80°C until analysis. Plasma and urine samples were analyzed for drug concentrations using LC-MS/MS assay.

In vivo pharmacokinetic data analysis

Plasma concentration–time profiles after intravenous administration were analyzed by noncompartmental analysis using Phoenix-WinNonlin 6.2 (Pharsight Corporation). The peak plasma concentration (C_{max}) was obtained by visual inspection of the plasma concentration–time curves. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$, where λ_z is the terminal phase rate constant. The area under the plasma concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule with extrapolation to time infinity. Volume of distribution (Vd) was calculated as ratio of the area under the first moment curve ($AUMC_{0-\infty}$) time dose divided by the square of $AUC_{0-\infty}$. The plasma clearance (CL) was calculated using the equation $CL = Dose_{iv}/AUC_{0-\infty,\ iv}$, where $Dose_{iv}$ and $AUC_{0-\infty,\ iv}$ are the intravenous dose and corresponding area under the plasma concentration–time curve from time 0 to infinity, respectively. The fraction (f_e) of the test compound excreted in urine was calculated as the cumulative amount of dose excreted unchanged in urine divided by the administered dose of the test compound. All values are presented as mean results from five animals.

Mouse challenge

All experiments involving animals were performed with prior approval of and in accordance with the guidelines of the St. Jude Institutional Animal Care and Use Committee. The St. Jude laboratory animal facilities have been fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the Department of Health and Human Services publication *Guide for the Care and Use of Laboratory Animals*. All mice were maintained in BSL2 facilities, and infectious challenges were undertaken while the mice were under inhaled isoflurane (2.5%) anesthesia. Mice were inoculated intranasally with 2.5×10^7 colony-forming units (CFUs) of serotype 2 pneumococcus strain D39x, engineered to express luciferase, or 3.0×10^7 CFUs of strain TIGR4 in 30 μl of PBS (*46*). Starting at 18 hours after challenge, a time when the bacteria have begun translocation into the bloodstream, mice were dosed twice daily with PlasmaLyte (vehicle control), ampicillin (2.5, 5, and 100 mg/kg), compound 1 (2.5, 5, 25, and 50 mg/kg), or spectinomycin (5, 25, and 50 mg/kg) via subcutaneous injection. Treatments were performed in a blinded manner with the compounds being drawn by one individual (unblinded) and then administered in a blinded fashion to the remaining groups. Xenogen imaging and blood CFU for bacterial burden were determined at 24 hours after challenge and every 24 hours thereafter. Blood titers were collected from treatment groups according to a key (that is, group 1, etc.) and counted before being unblinded.

Statistical analysis

Mantel-Cox (log-rank test) was used to determine statistical significance of mouse survival data. Mann-Whitney test was used to determine significance between bacterial burden in different treatment groups. In both analyses, $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

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Method S1. Chemical syntheses.
Method S2. Chemical stability studies.
Scheme S1. Synthesis of *R*-3′-aminomethyl-3′-hydroxy spectinomycins.
Scheme S2. Synthesis of N-benzyl aminomethyl spectinomycin S-isomer controls.
Fig. S1. Multiple sequence alignment of RpsE (protein S5).
Fig. S2. In silico analysis of the spectinomycin binding pocket in amSPC-resistant mutants.
Fig. S3. Computation analysis of the amSPC mutant binding site.
Fig. S4. Susceptibility of *L. pneumophila* to spectinomycin and amSPCs.
Fig. S5. Susceptibility of *C. trachomatis* to spectinomycin and amSPCs.
Fig. S6. Chemical stability of spectinomycin and compound 1.
Fig. S7. Efficacy in a mouse model of *S. pneumoniae* TIGR4 infection.
Fig. S8. Efficacy trial comparing matched doses of compound 1 and ampicillin.
Table S1. Activity against aerobic Gram-negative pathogens and *M. tuberculosis*.
Table S2. Testing against mammalian ribosomes.
Table S3. Lead profiling of compound 1.
Table S4. Sensitivity of amSPC-resistant clones to various classes of antibiotics.
Table S5. Antibacterial spectrum of activity for previously reported spectinomycins.
Table S6. Chemical stability of spectinomycin and compound 1.

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Acknowledgments: We thank R. Thomas for his advice, J. Scarborough for high-resolution mass spectroscopy data generation and processing, and T. Matt and T. Janusic for help with the ribosomal assays. We are also grateful to E. Tuomanen from St. Jude Children's Research Hospital (SJCRH) for critical reading of the manuscript. **Funding:** Supported by NIH grants AI090810 and AI111449, and the American Lebanese Syrian Associated Charities (ALSAC), SJCRH. **Author contributions:** R.E.L., S.L.W., and J.L. designed the compound series. S.L.W. synthesized the compounds. R.E.L., J.W.R., B.M., E.C.B., and D.F.B. designed the experiments. Z.Z. and D.F.B. performed in silico analyses. D.S. and S.D. performed ribosome inhibition assays. A.P.S. tested compounds for mammalian cytotoxicity testing and hemolysis. D.F.B. performed extracellular bacterial susceptibility testing and mutant analysis.

Y.M.A. and R.J.B. performed chlamydial testing. D.B.M. and C.R. performed pharmacokinetic and in vitro chemical stability analyses. J.W.R. conducted efficacy trials. D.F.B., R.E.L., R.B.L., E.C.B., B.M., and S.L.W. wrote the paper. All authors critically reviewed the paper. **Competing interests:** St. Jude Children's Research Hospital filed a provisional patent application related to this work in 2013 and an international application in 2014. There are no issued patents related to this work. R.E.L., S.L.W., D.F.B., Z.Z., J.L., and J.W.R. hold intellectual property rights ownership associated with the aminomethyl spectinomycin series. R.E.L. has been a paid consultant to AstraZeneca and to Microbiotix Inc., which has licensed this technology, although on topics not related to the work in this study and before the disclosure of this compound series. **Data and materials availability:** Data and materials not presented in the article or Supplementary Materials may be obtained by contacting R.E.L.

Submitted 4 September 2014
Accepted 18 March 2015
Published 20 May 2015
10.1126/scitranslmed.3010572

Citation: D. F. Bruhn, S. L. Waidyarachchi, D. B. Madhura, D. Shcherbakov, Z. Zheng, J. Liu, Y. M. Abdelrahman, A. P. Singh, S. Duscha, C. Rathi, R. B. Lee, R. J. Belland, B. Meibohm, J. W. Rosch, E. C. Böttger, R. E. Lee, Aminomethyl spectinomycins as therapeutics for drug-resistant respiratory tract and sexually transmitted bacterial infections. *Sci. Transl. Med.* **7**, 288ra75 (2015).

Science Translational Medicine

Aminomethyl spectinomycins as therapeutics for drug-resistant respiratory tract and sexually transmitted bacterial infections

David F. Bruhn, Samanthi L. Waidyarachchi, Dora B. Madhura, Dimitri Shcherbakov, Zhong Zheng, Jiuyu Liu, Yasser M. Abdelrahman, Aman P. Singh, Stefan Duscha, Chetan Rathi, Robin B. Lee, Robert J. Belland, Bernd Meibohm, Jason W. Rosch, Erik C. Böttger and Richard E. Lee

Sci Transl Med **7**, 288ra75288ra75.
DOI: 10.1126/scitranslmed.3010572

Teaching an old antibiotic new tricks

More and more cases of gonorrhea no longer respond to standard antibiotic treatment, leading the CDC (U.S. Centers for Disease Control and Prevention) to classify *Neisseria gonorrhoeae* as an urgent threat. New antibiotics are urgently needed to treat this and other emerging drug-resistant pathogens. To this end, Bruhn and Waidyarachchi *et al*. have taken a second look at an old group of antibiotics, the spectinomycins, a class of drugs that inhibit bacterial protein synthesis but do not kill many types of pathogens. By carefully mapping how the drug binds to the ribosome structure, the authors determined that *N*-benzyl–substituted spectinomycins should be able to inhibit the ribosomes of a broad spectrum of bacteria that produce disease. And indeed, this new series potently inhibited bacteria that cause respiratory illness (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* , and *Moraxella catarrhalis*) and sexually transmitted disease (*N. gonorrhoeae* and *Chlamydia trachomatis*). Their pharmacokinetics properties were promising, and assays showed that they are unlikely to cause adverse reactions. These new spectinomycins are active against drug-resistant forms of *S. pneumoniae* and cure mice of fatal pneumococcal pneumonia and sepsis, an encouraging result for the eventual use of these drugs for human infection.

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