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Aminomethyl Spectinomycins as Novel Therapeutics for Drug Resistant Respiratory Tract and Sexually Transmitted Bacterial Infections

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Abstract

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 novel series of *N*-benzyl substituted 3'-(*R*)- 3'-aminomethyl-3'-hydroxy spectinomycins was developed based on 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 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 leads 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. In addition to improved antibacterial potency, compounds also demonstrated no mammalian cytotoxicity,

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Author contributions: R.E.L., S.L.W., and L.J. designed the compound se ries. 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., and S.L.W. wrote the paper. All authors critically reviewed the paper.

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improved microsomal stability, and favorable pharmacokinetic properties in rats. The lead compound from the series, compound 1, exhibited excellent chemical stability, which was superior to spectinomycin and had no significant interaction with a panel of human receptors and drug metabolism enzymes suggesting low potential for adverse reactions or drug-drug interactions *in vivo*. Compound 1 was active *in vitro* against a panel of penicillin, macrolide, and cephalosporin resistant *S. pneumoniae* clinical isolates and cured mice of fatal pneumococcal pneumonia and sepsis at a dose of 5 mg/kg. Together, these studies indicate *N*-benzyl aminomethyl spectinomycins possess suitable properties for further development as novel antibacterial agents to treat drug resistant respiratory tract and sexually transmitted bacterial infections.

INTRODUCTION

The continuous rise in the prevalence of antibiotic resistance amongst 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 the 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 *Neisseria gonorrhoeae* is weak, yet the antibiotic's high safety margin permits use of 2-4 gram intramuscular injections (9-12). Attempts to develop spectinomycin analogues 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 as the third generation cephalosporins and second generation macrolides in development and use were judged superior at the time. These efforts validated the potential to modify the spectinomycin core to obtain more potent generations of this antibiotic (13-16).

We 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 anti-tubercular agents active against MDR/XDR tuberculosis that have robust activity in acute and chronic models of tuberculosis infection (17). Key to the

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antituberculosis activity of lead spectinamides was the introduction of 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 *M. 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 analogues 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 anti-tubercular 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 demonstrate this pocket is also accessible with *N*- benzyl substitutions to the amino methyl side chain of the amSPCs. Thus, the aim for the current study became to apply this knowledge to the amSPC core to develop novel *N*-benzyl aminomethyl spectinomycins with favorable pharmacological properties and improved antibacterial activity to treat drug resistant bacterial infections (Fig. 1E).

RESULTS

Computation modeling of *N*-benzyl substituted aminomethyl spectinomycins

To guide our design 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 *E. coli* ribosome, a homology model of the *Streptococcus pneumoniae* ribosome was built as a representative gram-positive spectinomycin binding site (Fig. 2) (7). Within a 15Å 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, Fig. S1). The four RpsE protein loop residues closest to the spectinomycin binding site (V24, K25, G26, 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 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.

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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 (MD) simulation upon changing the corresponding outer RpsE loop residues in *E. coli* to *S. pneumoniae* sequence (S21T, T23V, I329R, F30L) as 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.

Synthesis of *N*-benzyl aminomethyl spectinomycins

Based on our *in silico* modeling results, targeted 3' *R*-isomer amSPCs (compounds 1-4) were designed and synthesized from spectinomycin in a five step sequence according to the procedure of Thomas, utilizing optimized protocols for the reductive amination and Cbz deprotection steps (Method S1, Scheme S1) (20, 21). The corresponding 3' *S*-isomer controls (compounds 5-6) were generated by an identical approach (Scheme S2) with exception of the key hydrocyanation step, which was performed using acetone cyanohydrin in methanol in 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. However, a full description of the Structure-Activity-Relationship (SAR) against multiple bacterial pathogens is beyond the scope of this manuscript. Herein, we focus this first report on compounds with the best clinical potential as judged by their balance of improved antibacterial activity, favorable PK 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, Table S1) (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} 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 chloride (compound 4) as similar substitution patterns were found favorable in the spectinamide series and to also block a site of potential oxidative metabolism. Introduction of the halide substituents were well tolerated, with chloro (compound 4) producing the most potent protein translation inhibitor. The corresponding *S*-isomers of compound 1 (compound 5) and 3 (compound 6) were synthesized (Scheme S2) and tested. As predicted from modeling experiments, the *S*-isomers had ablated protein synthesis inhibition compared to their corresponding *R*-isomers (Table 1). Importantly, 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 16S rRNA helix 34 and RpsE

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between bacterial and mammalian ribosomes. Both cytosolic and mitochondrial mammalian ribosomes have significant nucleotide polymorphisms in the 16s rRNA helix 34 when compared to the conserved bacterial sequence, which we have previously shown 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 non-specific activity towards 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 non-cytotoxic against mammalian cells and non-hemolytic 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 -negative pathogens. (Table 1 and Table S1). The MICs of amSPCs improved against gram positive pathogens when compared to spectinomycin. The greatest potency was seen for *Streptococcus* species with MICs for ribosomally active compounds ranging from 0.8 – 12 μg/mL. Substantial improvement in activity was also seen for *E. faecalis* where addition of the unsubstituted aminomethyl benzyl group decreased the MIC from 100 to 25 (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 enterobacteriacea, pseudomonad, 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

Streptococcus pneumoniae is an important respiratory pathogen causing pneumonia, otitis media, and meningitis, for which drug resistance is an increasing 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). Importantly, 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 (PBP). To further test for cross-resistance

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potential, *Streptococcus 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 for 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, clones remained weakly susceptible to the parent spectinomycin. To rationalize this result, mutant genomes were sequenced and while no mutations were identified in the RNA helix 34, a mutation in RpsE (G27R) was identified (Figure S2). *In silico* docking of spectinomycin and compound 1 predicted this mutation to exert greater impact on the binding of compound 1 as compared to spectinomycin (Figure S3). This is in agreement with the drug sensitivity observed and further indicated that the antibacterial activity of amSPCs in a consequence of ribosomal inhibition. The susceptibility of drug-resistant pneumococci to amSPCs and lack of cross resistance with compound 1 *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 treatment of atypical and severe respiratory diseases. Since 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 that is susceptible to spectinomycin (MIC of 6 μg/mL). The amSPCs maintained activity of the parent spectinomycin towards 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 due to its resistance to common antibiotics. We were able to improve potency from 25 μg/mL 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 20-40 mm (Table 2, Fig. S2), suggesting they overcome intrinsic resistance. Together these data demonstrate that amSPCs have superior activity to spectinomycin against common bacterial respiratory pathogens.

Neisseria 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 United States market was discontinued in 2005. However, isolates resistant to all available treatments including the cephalosporin ceftriaxone have recently arisen and now pose a tremendous

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threat to public health worldwide (25, 26). This underscores the dire need for new treatments for this sexually transmitted disease (STD). The amSPCs were tested for activity against *Neisseria gonorrhoeae* and closely related *Neisseria meningitidis* (Table 3). Compound 4 demonstrated the best activity of the compounds in this set, producing a zone of inhibition of 20-22 mm, double the 10-11 mm zones produced by spectinomycin (Fig. 3). Since 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. S3). Up to 100 μg/mL of spectinomycin failed to reduce intracellular inclusions in *C. trachomatis*-infected monolayers, however, 12 μg/mL of compounds 1 and 4 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 (data not shown). This is in agreement with cytotoxicity testing and indicates that reduction in intracellular *C. trachomatis* produced by compounds 1 and 4 arises from their anti-chlamydial 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 re-synthesized two of the most potent alkyl analogues previously reported by UpJohn Company as having improved antibacterial activity. These alkyl-substituted analogues showed no improvement in potency over spectinomycin towards the bacterial therapeutic targets discussed in this manuscript, with the exception of *Legionella pneumophila* (Table S5). The antibacterial spectrum for the amSPCs is also notably different than 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 TB 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 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 (43-63%), 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 all compounds had high metabolic stability, either similar to (compound 1) or better than (compound 3 and 4) spectinomycin.

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The chemical stability of compound 1 was examined in comparison to spectinomycin at pH 2, 7, and 9 (Table S6, Fig. S4) 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 4 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.

Since *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 - 21.5 μg/ml and an area under the curve of 17.4 – 19.4 μg h/ml. The compounds exhibited biexponential plasma concentration-time profiles with a half-life of 1.12-1.99 h at therapeutically relevant concentrations above the MIC, which is longer than the 0.75 h observed for spectinomycin in rats. As clearance was similar to spectinomycin for all compounds, the longer half-life observed for compounds3 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 58-82% 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 anti-bacterial drugs and were suitable for progression into *in vivo* efficacy trials.

amSPCs rescue mice from a lethal pneumococcal infection

amSPCs 1, 3, and 4 were tested for anti-pneumococcal activity in mice infected with *S. pneumoniae* strain D39, which causes pneumonia and bacteremia. Compounds were administered subcutaneously twice daily (BID) to infected mice 18 hours post intranasal challenge, a time when mice have developed both pneumonia and bacteremia (32). Mice receiving compound 1 or 4 at the lowest dose (5 mg/kg) all survived whereas all mice receiving vehicle and spectinomycin controls died or were humanely euthanized by 120 hours post challenge (Fig 4A,C). Compound 3 improved survival for 3 of 5 mice at 5mg/kg, but was not as protective as compounds 1 and 4 (Fig. 4B). The bacterial burden in the blood 48 hours post-challenge was reduced significantly ($p<0.05$) in groups receiving compound 1 or 4 compared to the spectinomycin and vehicle controls (Fig 4 D,F). Clearance of the infection was also evident in the bioluminescent images of mice at 72 hours post challenge, which showed systemic bacterial infection in both the vehicle and spectinomycin groups whereas mice administered compounds 1 and 4 cleared the infection below detectable limits (Fig 4G-I).

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To determine how the efficacy of compound 1 compared to ampicillin, an antibiotic used for *S. pneumoniae* infections, the efficacy of matched doses of compound 1 and ampicillin was tested (Figure S9). Mice administered either compound 1 or ampicillin (matched at 2.5 or 5 mg/kg) all survived the 9 day experiment, whereas 4 of the 5 mice administered the carrier died by day 5 post infection. Within 48 hours post infection, bacterial titers were reduced by more than 3 logs in treatment groups and barely above the limit of detection. Reduction of bacterial burden provided by the compound 1 treatment groups 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. S8). 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, while 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

In this current report, 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 analogues: drug resistant sexually transmitted and drug resistant respiratory bacterial pathogens, including several highlighted the Center for Disease Control and Prevention's 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 ADME properties. Compounds in this series have a mechanism of action distinct from that of other antibiotics, including other protein synthesis inhibitors, are highly selective towards 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 whole cell activity was not related to increased ribosomal affinity, suggesting the molecular basis for their superior MIC is extra-ribosomal. Studies involving the 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 amSPC also have greater accumulation within target bacteria and

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that avoidance of species-specific drug-efflux mechanisms is also key to the activity of this series.

The Centers for Disease Control and Prevention (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* H041strain, which is causing much clinical concern, spectinomycin is one of the few therapeutic choices remaining (33). In the current study, the spectinomycin analogues 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 grams required currently for therapy with spectinomycin for gonorrhea. Unfortunately, pre-clinical testing of antigonorrheals is hindered by poor colonization of lower vertebrates (including rodents) and the high costs and ethical issues of using non-human primate models for gonor-rhea infection (34). Thus, our pre-clinical development of the amSPCs for this indication will likely employ a PK/PD 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 anti-gonorrheal candidate AZD0914, which has been given Fast Track status by US Food and Drug Administration (FDA) (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 betalactams due to increasing clindamycin and macrolide resistance (36-38). Since *S. pneumoniae* is a predominant cause of childhood upper respiratory tract infections and treatment is compromised by resistance to macrolides and betalactams, we focused our *in vivo* efforts on this pathogen. We demonstrate that amSPCs possess potent *in vivo* activity capable of clearing high burden, lethal *S. pneumoniae* infections at low doses. *mefE* efflux was our greatest concern with respect to preexisting cross resistance mechanism 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 treatment of the respiratory pathogen*Mycobacterium 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 associated bacterial pneumonia for which there are other therapeutic options.

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We have previously demonstrated that spectinomycin selectivity towards bacterial ribosomes and avoidance of human mitochondrial ribosomes, despite the bacterial origin of this organelle, is explained by sequence variations within RNA and associated RpsE protein portion of the spectinomycin binding site. Amongst 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, as 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 as the chemically reactive keto group within the 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 MIC values alone would suggest. This is clearly in part due to their good ADME properties, as we have observed with the spectinamide series.

Our recent studies have re-evaluated the potential for synthetic modification of the spectinomycin core using new ribosome binding information and prior chemical literature from the 1970-1980s producing the antitubercular spectinamides and the second generation amSPCs reported herein. These studies each generated chemically distinct series of spectinomycins with non-overlapping disease indications. The spectinamides have excellent activity specific to the *Mycobacterium tuberculosis* complex whereas the *N*-benzyl substituted amSPCs have robust activity against resistant respiratory tract and sexually transmitted bacterial pathogens. These studies highlight the tremendous potential of modifying the 3' position of the exterior facing C-ring in spectinomycin to modulate antibacterial spectrum of activity while maintaining ribosomal inhibitory potency, to provide potential therapeutic agents suitable to treat drug resistant bacterial infections. Further preclinical development of the amSPCs is underway in our laboratories.

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 co-crystal structure of spectinomycin bound to the 30s ribosome from *E. coli* (PDB id: 2QOU) was used for construction of a homology model of the spectinomycin binding site from S. *pneumoniae* using the same approach we have recently applied to the *M. tuberculosis* ribosome (17). Primary sequence variances within a 15Å-truncated sphere centered at the binding site A1081G and S22T, T24V, I30R, F31L) were identified. Mutations were then introduced by the "Mutate Residues" script implemented in Maestro,

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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 5ns molecular dynamics simulations which was performed as described previously (17).

Ribosomal inhibition assays

S-30 extracts or purified ribosomes were used in translation reactions of luciferase mRNA as described previously (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 70S ribosomes, 4 μg F-luc mRNA, 40% (vol/vol) S100 extract, 200 μM amino acid mixture, 24 units of RiboLock (Fermentas), 0.4 mg/ml of tRNAs, and 12 μl of commercial S30 Premix without amino acids (Promega). Following 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 50% inhibitory concentration (IC50), i.e., the drug concentration that results in 50% inhibition of luciferase synthesis. When purified bacterial ribosomes were used, these were isolated from *Mycobacterium smegmatis* to permit comparison with our previously published data for spectinomycin derivatives. We have shown previously that data for inhibition of *E. coli* and *M. smegmatis* correlate and note that the expression the 16s 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 muta-genesis.

Bacterial growth

Bacillus subtilis (ATCC 23857), *Enterococcus faecalis* (ATCC 33186), *Staphylococcus aureus* (ATCC 29213), MRSA *Staphylococcus aureus* (NRS70), *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (strain K12), *Klebsiella pneumoniae* (ATCC 33495), *Pseudomonas aeruginosa* (PA01), *Stenotrophomonas maltophilia* (ATCC 13637) and *Burkholderia cepacia* (ATCC 25416) were maintained in Mueller Hinton broth or on agar plates. *Streptococcus pneumoniae* (strain R6) and *Streptococcus pyogenes* (ATCC 700294) were cultured in Mueller Hinton in the presence of 10% (volume/volume, final) defibrinated, lysed horse blood. *Listeria monocytogenes* (NS512) and *Moraxella catarrhalis* (ATCC 25238) were cultured in Brain Heart Infusion (BHI). *Neisseria gonorrhoeae* (ATCC 49226), *Neisseria meningitidis* (ATCC 13077), and *Haemophilus influenzae* (ATCC 49247) were maintained on GC agar supplemented with 2% (volume/volume) IsovitalX and 1% (weight per volume) hemoglobin. *Legionella pneumophila* (ATCC 33153) was maintained on CYE supplemented with BCYE. Agar plates for *Neisseria gonorrhoeae, Neisseria meningitidis,* and *Haemophilus 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. *Mycobacterium tuberculosis* (H37Rv) was cultured in Middlebrook 7H9 broth (Difco Laboratories, MI, USA) supplemented with 10% albumin–dextrose complex and 0.05% (v/v) Tween80.

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Liquid MIC testing

Minimum inhibitory concentrations (MICs) were determined in appropriate media (indicated above) according to Clinical Laboratory Standards Institute (CLSI), using two fold serial dilutions in 96-well plates (Thermo Scientific) starting at 200 μg/mL drug (41). Plates were incubated in ambient air at 37°C and MICs recorded after 16-20 hours of incubation, except for *M. tuberculosis* MICs which were recorded after 7 days of incubation.

Disc diffusion assays

A 4 μl aliquot of 10 mg/mL stock solution (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 2 biologically independent experiments.

Hemolysis assays

Defibrinated sheep blood purchased from Colorado Serum Company was centrifuged gently at 350 rcf for 20 minutes. The two upper layers (buffy coat and plasma) were discarded, and erythrocytes washed three times in sterile phosphate-buffered solution (PBS), pH 7.4. After the third wash, cells were diluted to a final v/v concentration of 5% in PBS. Two fold 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 transferred to an optically clear, flat, white wall 96-well plate taking care not to resuspend unlysed cells. Absorbance of supernatants was then read at an optical density (OD) of 540 nm. Percent hemolysis was calculated using the formula % lysis = (OD540 of sample – OD540 of blank) / (OD540 of positive control).

Cytotoxicity testing

Vero (kidney epithelial cells; ATCC CCL-81) monolayers were trypsinized and 5000 cells/well were seeded (10-15% confluence) into white-wall, flat bottom 96 well microwell plates (Corning) using enriched Dulbecco's Modified Eagle's Medium (Hyclone: DMEM/ High Glucose) 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 media was then replaced with media 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] vs. Response-variable slope (four parameters) - symmetrical equation, in GraphPad Prism version 6 [GraphPad Software, La Jolla California USA, www.graphpad.com]. For each experiment, compounds were tested in duplicate. IC_{50} values presented are the range of two biologically independent experiments.

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Chlamydial culture and susceptibility testing

Chlamydiatrachomatis serovar L2 (strain 434/Bu) was grown in HeLa 229 cells cultivated at 37°C with 5% CO_2 in high glucose-containing DMEM (Cellgro, Mediatech) supplemented with 10% heat-inactivated FBS. Elementary bodies (EBs) were purified on density gradients of RenoCal-76 (Bracco Diagnostics, NJ, USA) 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 6-well plates containing DMEM with 10% FBS. The monolayers were pre-treated with DEAE-Dextran (30 μg/ml) and infected at a multiplicity of infection (MOI) of 0.5. Experimental compounds and reference antibiotic spectinomycin (originally dissolved at 100 mg/mL in 100% DMSO) were diluted directly in culture media 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. Levels of infection were estimated by counting the number of mCherry positive inclusions per field (44).

In vitro pharmacokinetic studies and analysis

Plasma protein binding was determined by equilibrium dialysis at 37°C using the RED device (Thermo Scientific, Rockford, IL). Microsomal metabolic stability of aminomethyl spectinomycin compounds was assessed in pooled rat liver microsomal preparations (Cellzdirect, Austin, TX) by monitoring disappearance of the parent compound over an incubation period of 90 minutes as described previously (45). Drug concentrations were determined by LC-MS/MS assay and calculations were as described previously (31).

In vivo pharmacokinetic studies

Catheterized male *Sprague-Dawley* rats (femoral vein for drug administration and jugular vein for blood sample collection) weighing approximately 200-225 g were obtained from Harlan Bioscience (Indianapolis, USA). Animals were kept on a 12 h light/ dark cycle with access to food and water *ad ibitum*. Animal studies were conducted according to the guideline of 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 dissolvedin PlasmaLyte and administered intravenously (IV) to a group of five rats (n= 5) at a dose of 10 mg/kg. Serial blood samples (approx. 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 h post-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 0-6, 6-12, 12-24, 24-36 and 36-48 h post-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 following IV administration were analyzed by non-compartmental analysis using Phoenix-WinNonlin 6.2 (Pharsight Corporation, Mountain View, CA). The peak plasma concentration (C_{max}) was obtained by visual inspection of the

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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 ($AUMC0-_{\infty}$) time dose divided by the square of $AUC0-_{\infty}$. 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 IV 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 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 (DHHS) publication, Guide for the Care and Use of Laboratory Animals. All mice were maintained in BSL2 facilities, and infectious challenges undertaken while the mice were under inhaled isoflurane (2.5%) anesthesia. Mice were inoculated intranasally with 2.5×10^7 colony forming units (CFU) 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 post 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, 100 mg/kg), Compound 1 (2.5, 5, 25, 50 mg/kg), of spectinomycin (5, 25, 50 mg/kg) via subcutaneous injection. Xenogen imaging and blood CFU for bacterial burden were determined at 24 hours post challenge and every 24 hours thereafter.

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, $p<0.5$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

A novel series of spectinomycin analogues with potency against drug resistant respiratory tract and sexually transmitted bacterial pathogens was successfully designed and developed using a structure-based approach and validated *in vitro* and *in vivo*.

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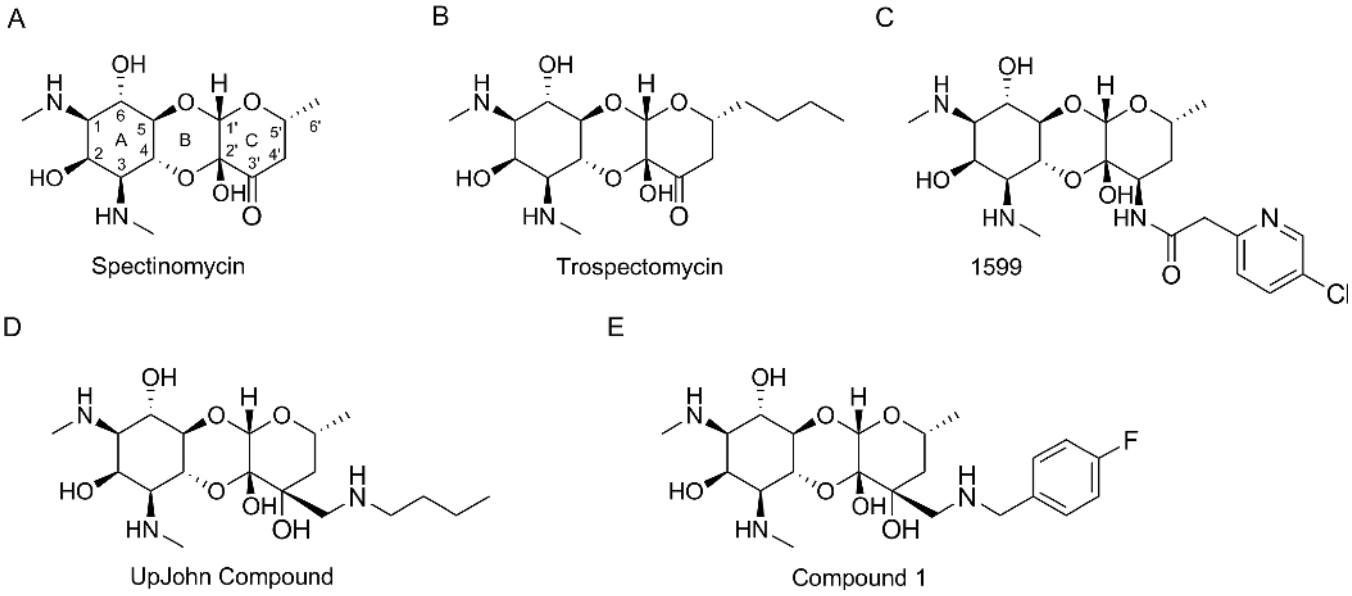

Figure 1.

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

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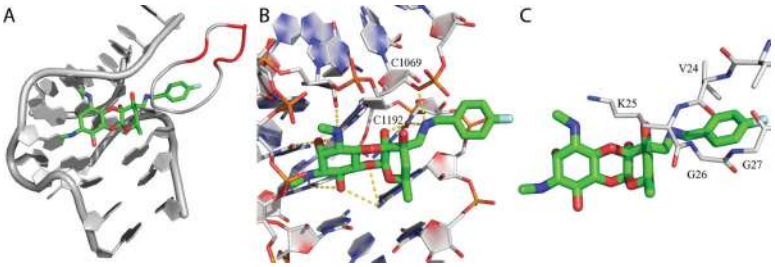

Figure 2.

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 30S 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. The homologous *E. coli* RpsE positions are given in parentheses.

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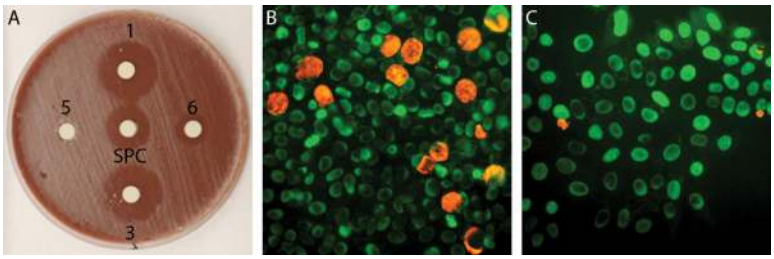

Figure 3.

Activity against *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. (**A**) Zone of inhibition testing for *Neisseria gonorrhoeae*. A representative image of disc diffusion assays for *N. gonorrhoeae* is shown. 40 μg of compound dissolved in DMSO was applied to the disc. (**B – C**) Images of mCherry-expressing *Chlamydia trachomatis* infected monolayers treated with 12 μg/mL of spectinomycin (**B**) or Compound 1 (**C**).

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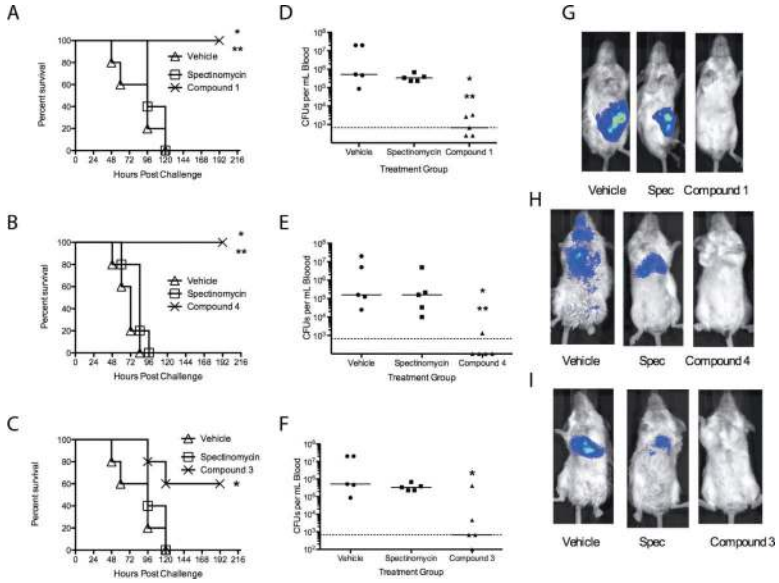

Figure 4.

amSPCs mediate more effective protection than spectinomycin from invasive pneumococcal challenge (5 mg/kg BID dose beginning 18 hours post challenge with *S. pneumoniae* D39x). (**A-C**) Overall survival of mice receiving vehicle control or the indicated compounds (n=5 mice per group). (**DF**) Bacterial burden in the blood 48 hours post-challenge. (**G-I**) Representative bioluminescent images of mice at 72 hours post challenge. Single asterisks (*) indicate $p < 0.05$ when compared to vehicle control group. Double asterisks (**) indicate $p < 0.05$ when compared to spectinomycin control. Statisitical significance was determined using log rank test (Mantel-Cox) for survival data and Mann-Whitney test for bacterial burden data.

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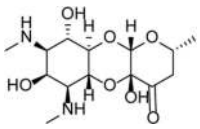
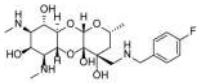
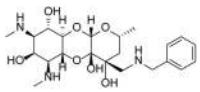
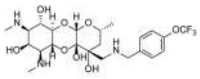
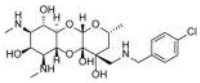
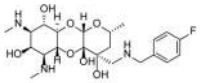
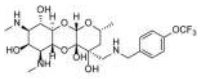
Table 1

Ribosomal, Cytotoxic and Gram positive Activity of Methylspectinomycin Analogues

Structure	IC_{50} µg/ml		MIC µg/ml[c]						
	Ribo.[a]	Vero[b]	*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	-

Organisms abbreviated above are as follows: 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).

[a] Ribosomal (Ribo.) IC_{50} values were determined using ribosomes purified from *M. smegmatis*.

[b] Cytotoxicity IC_{50} was determined *in vitro* using mammalian cells (Vero) and the average of two separate experiments is presented, with standard error of mean provided in parenthesis.

[c] MIC results presented are the range of two biologically independent experiments. Dashes (-) indicate where values have not been determined.

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Table 2

Anti-Pneumococcal Activity of Select amSPCs against Drug Sensitive and Resistant 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

MIC testing for amSPC activity against a panel of *S. pneumoniae* isolates. Results of a single experiment are presented.

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Table 3

Zone of Inhibition Testing against Fastidious Gram Negative Pathogens

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

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.

Organisms abbreviated above are as follows: *Ng, Neisseria gonorrhoeae* (ATCC 49226); *Nm, Neisseria meningitidis* (ATCC 13077); *Hi, Haemophilus influenzae* (ATCC 49247) and *Lp, Legionella pneumophila* (ATCC 33153).

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Table 4

Pharmacokinetic Parameters for Select amSPCs

Compound	Protein Binding	Microsomal Stability	IV Pharmacokinetics (Dose: 10 mg/Kg)			
	% Bound	$t_{1/2}$ (hr)#	$T_{1/2}$ (hr)	Vd (L/Kg)	CL (L/hr/Kg)	fe
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)

Parameters are expressed as mean. Values in the parenthesis indicate % CV.

Abbreviations: t½, half life; Vd, volume of distribution; CL, clearance; fe, fraction excreted unchanged in urine.

*Spectinomycin values are from reference (31) and included for comparison.

#t½ is based on decline of plasma concentration in the therapeutically relevant concentration range.