Published in final edited form as:

Eur J Med Chem. 2019 February 01; 163: 381–393. doi:10.1016/j.ejmech.2018.11.022.

Synthesis, Antimicrobial Activity, Attenuation of Aminoglycoside Resistance in MRSA, and Ribosomal A-site Binding of Pyrene-Neomycin Conjugates

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Abstract

The development of new ligands that have comparable or enhanced therapeutic efficacy relative to current drugs is vital to the health of the global community in the short and long term. One strategy to accomplish this goal is to functionalize sites on current antimicrobials to enhance specificity and affinity while abating resistance mechanisms of infectious organisms. Herein, we report the synthesis of a series of pyrene-neomycin B (PYR-NEO) conjugates, their binding affinity to A-site RNA targets, resistance to aminoglycoside-modifying enzymes (AMEs), and antibacterial activity against a wide variety of bacterial strains of clinical relevance. PYR-NEO conjugation significantly alters the affinities of NEO, for bacterial A-site targets. The conjugation of PYR to NEO significantly increased the resistance of NEO to AME modification. PYR-NEO conjugates exhibited broad-spectrum activity towards Gram-positive bacteria, including improved activity against NEO-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Graphical Abstract

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Keywords

RNA; Aminoglycoside; MRSA; Neomycin; Ribosome

Introduction

Multidrug-resistant pathogens have been rapidly evolving worldwide over the past few decades, greatly reducing the effectiveness of current drugs.¹ Targeting nucleic acids with small molecules can be an effective therapeutic strategy for treating bacterial pathogens by disrupting DNA synthesis, RNA synthesis, and/or interfering with translation. Inhibition of protein synthesis is a key feature of many antimicrobials. Aminoglycosides are a class of small molecules that bind to nucleic acids (RNA) and have been well established therapeutics since the 1940s.² Aminoglycoside binding occurs through electrostatic, van der Waals, and hydrogen bonding interactions between the positively-charged amino sugars and the negatively-charged nucleic acid (RNA) backbone and nucleotide bases. The aminosugars bind tightly to the A-site 16S ribosomal RNA (rRNA) with high affinity and cause translational errors resulting in ultimate cell death in bacteria.^{3–4} A-form nucleic acid structures, as adopted by the RNA, exhibit deep and narrow major grooves, which has been shown to be the ideal structural motif for binding of aminoglycosides.^{5–9} Aminosugars can even bind DNA oligonucleotide duplexes such as 5'G₄C₄-3'DNA that has A-form characteristics, with a sub-micromolar binding affinities ($K_a \sim 10^6$ - 10^7 M⁻¹),¹⁰ much higher binding affinity than for predominantly B-form, AT-rich sequences.¹⁰ Neomycin B (NEO) is an attractive compound to study nucleic acid binding since it binds with a higher affinity to a variety of nucleic acid shapes compared to other aminoglycosides and can be easily modified with different functionalities without loss of the RNA binding amino groups. This understanding has led to recognition of alternate nucleic acid sequence target sites for drug development, including the B-form DNA.¹¹ Through functionalization of the 5''-hydroxy group on ring III of NEO with intercalators, DNA minor groove binders, and through dimerization of NEO or other comparably functionalized aminoglycosides, many different binding sites for aminoglycoside-based compounds have been established.^{12–15} Functionalization of the 5''-OH of NEO has been shown to lead to higher binding affinities to different aminoglycoside binding sites, decreased resistance to aminoglycoside-modifying enzymes (AMEs), and improved RNA selectivity and antibacterial effects.^{16–17} In bacteria,

the aminoglycoside binding-site is the bacterial 16s rRNA A-site. As bacterial resistance to traditional antibiotics continues to spread, novel aminoglycoside modifications offer considerable value in identifying effective leads that can circumvent bacterial resistance mechanisms. Modification of NEO through functionalization of the 5''-OH position represents one pathway of evading potential antibiotic resistance mechanisms while maintaining comparable rRNA affinity and specificity of NEO to its target binding site.

Pyrene (PYR) and its derivatives are formed through combustion of organic compounds and are useful in industry and biochemical applications used to make plastics, pesticides, dyes, and fluorescent molecular probes. PYR is a relatively weak, non-specific nucleic acid intercalator that can be easily derivatized. PYR-NEO conjugates can be rapidly synthesized with various linker lengths through amide and thiourea couplings. Therefore, we postulated that PYR-NEO conjugates would provide a new class of NEO derivatives that exhibit differential affinities to the bacterial and mammalian RNA A-sites, compared to NEO, in addition to altered resistance and antibacterial effects.

In this report, we present the synthesis, rRNA A-site binding characteristics of a series of PYR-NEO conjugates with varying linker lengths using fluorescein-NEO (F-NEO) displacement, their activity as AME substrates, and their antibacterial effects against Gram-positive and Gramnegative pathogens. Five rRNA A-site homologues were screened that included *Escherichia coli*, human cytosolic, mitochondrial, and mitochondrial mutant A-site rRNA models. IC₅₀ values were obtained to determine the efficacy of these conjugates compared to NEO. The conjugates were also assayed for antibacterial activity against a wide range of bacteria of clinical importance. We identified an optimal linker-length for PYR-derivatized NEO that invokes selectivity to *E. coli* A-site compared to human A-site targets. Most PYR-NEO conjugates were remarkably resistant to the action of AMEs on the NEO molecule, and subsequently showed much improved activity against NEO-resistant MRSA strains, as compared to NEO. These results affirm that functionalization of NEO with readily available and simple compounds is a viable strategy of developing new antibiotics that have the potential to overcome known antibiotic resistance mechanisms.

Results and discussion

Synthesis

The functionalization of the 5''-OH position on NEO was performed as previously reported,^{16,18} and the synthesis of the PYR-NEO conjugates **10-16** (Figure 1) is summarized in Scheme 1. The amine groups on rings I, II, and IV are critical for electrostatic binding of NEO to nucleic acids, so the preferred functionalization site chosen was the 5''-OH group on ring III. Conjugation of NEO with readily available starting materials, such as PYR activated esters, with simple derivatization techniques allows for rapid generation of conjugates, and many of these conjugates were prepared in only a few simple steps requiring mild reaction conditions.

Compounds **10-16** were prepared by reacting the appropriate pyrene carboxylic acid compounds **1-3** with *N*-hydroxysuccinimide to yield pyrene succinimidyl esters **4-6** of various linker lengths. This procedure afforded stable PYR intermediates that were purified

by ethyl acetate extraction and washing with deionized water. Compound **16** was prepared in multiple steps using a thiourea linkage. First, pyrene butyric acid was reacted with *N*-hydroxysuccinimide to yield the pyrene succinimide ester, with subsequent nucleophilic attack by 1,6-hexanediamine in large (40 mol) excess. This intermediate was then reacted with thiocarbonyldipyridone (TCDP) to afford the pyreneisothiocyanate **7**. Isothiocyanate **7** was then reacted with NEO amine **8** to afford the desired conjugate **16** after deprotection of Boc groups with an acid. The linker lengths of all conjugates are listed in Figure 2 in ascending order.

Characteristics of rRNA A-site targets

The A-site targets studied in this report exhibit stem-loop secondary structures. The secondary structures of the A-sites involved in the study are shown in Figure 2. The A-sites exhibit the highly conserved G-C, the non-canonical U-U, and A-A-A bulge regions. It is known that aminoglycoside contacts are made on the A-site from rings I and II in NEO in which ring I interacts with the A₁₄₀₈-A₁₄₉₃ in a near orthogonal fashion to rings II, III, and IV.¹⁹ The amino and hydroxyl groups in these positions make contacts that stabilize the aminoglycoside-rRNA complex. PYR has shown to be a weak nucleic acid intercalator, alone or when bound to an adjacent cationic, groove binding moiety.^{20–21} The conjugation of PYR was expected to provide enhanced binding affinity if the proper atomic linker between the III-ring of NEO and pyrene was utilized. Thus, it was expected that PYR-NEO would bind in this position with enhanced affinity to the target provided through intercalation or stacking of pyrene between the base pairs.

Affinity of the PYR-NEO conjugates to rRNA A-site targets

The screening technique used to study PYR-NEO binding to the A-site targets is a fluorescence-based F-NEO (Figure 3) displacement assay, developed previously.²² This technique uses a fluorescent NEO conjugate, which binds to the rRNA A-site specifically within grooves. The utility of F-NEO is that the fluorophore maintains pH sensitive fluorescent properties while the aminosugar exhibits binding properties similar to non-conjugated NEO.²³ Because of such properties, both moieties can be exploited using a simple fluorescence-based assay to measure the A-site binding affinities of molecules in direct comparison to NEO and fluorescein, respectively. As ligand is added to the bound RNA:F-NEO complex, F-NEO is displaced. F-NEO exhibits considerable fluorescence changes when bound to its target sequence compared to unbound F-NEO which is comparable to fluorescein. The fluorescence changes are directly related to binding or dissociation of F-NEO from the A-site by a competing ligand.

The series of PYR-NEO conjugates were screened against five RNA A-site homologues and the relative binding affinity was compared to NEO. The data shows some dependence of binding on the linker-length during conjugate binding (Table 1). Conjugates with linker lengths of 4-5 atoms bound poorly to all rRNA A-sites compared to NEO. However, as the linker length reached six atoms, binding affinity increased greatly and equaled the binding affinity of NEO in all A-sites.

PYR-NEO conjugate affinity to the A-site reached a maximum with a seven-atom linker length. As the linker length became greater between PYR and NEO, the affinity of the respective conjugate again reduced, though the percent reduction was not as great as observed for the smallest linkers. These results show that an optimum atomic linker length of seven atoms from the NEO 3' ring to the fused rings of PYR to achieve the highest binding affinity. It should be noted that the intermolecular interactions also depend on the shape of molecules, and kind of hetero atoms in the linkers. It is likely that the bioactive conformations are affected by the hydrophilicity and flexibility of the linker, in addition to the length of the linker atoms.

IC₅₀ of PYR-NEO conjugates to the A-site

IC₅₀ values represent the amount of ligand required to displace 50% of F-NEO from the nucleic acid target. The lower the IC₅₀, the lower the concentration of ligand required to displace F-NEO, which indicates a better binding ligand to the target sequence. IC₅₀ values were determined by titrating the A-site:F-NEO complex with small amounts of ligand until 50% F-NEO was displaced. Since F-NEO experiences a significant increase in fluorescence intensity as it is displaced from the A-site, differences in initial and final fluorescence values makes F-NEO a convenient probe for IC₅₀ determination. Listed in Table 2 are the IC₅₀ values for selected A-sites with PYR-NEO conjugates. The highest affinity conjugates are **12** and **13**, which required the lowest concentration of ligand to displace 50% of F-NEO. The optimum atomic linker length of conjugate to displace F-NEO is seven atoms, which mirrors the results obtained from A-site single point screening. For shorter linker lengths between pyrene and NEO (four and five atoms) atoms, IC₅₀ values are the highest in the group. However, as the linker length between PYR and NEO reaches seven atoms, the IC₅₀ values exhibit a 3 to 4-fold reduction.

Specificity comparison of PYR-NEO conjugates to A-site

NEO shows slight specificity to human RNA A-site targets compared to a model *E. coli* target RNA. A useful relationship to compare affinity of a ligand to bacterial A-site and human A-site is the selectivity factor. This value is a ratio of IC₅₀ values of ligand to human and *E. coli* target sequences, multiplied by a factor of 5.75.²⁵ Therefore, ligand values above 5.75 represent selectivity to bacterial A-site targets, and values below 5.75 represent selectivity to human A-site targets. NEO exhibits selectivity to human A-site targets compared to *E. coli* A-sites, with a selectivity factor of 5.02. Conjugation of pyrene to the 5'-OH position of NEO, however, changes selectivity from human A-sites to bacterial A-sites. Selectivity factor values of the PYR-NEO conjugates are given in Table 3. These values show that shorter linker lengths exhibit higher selectivity to bacterial A-site targets than PYR-NEO with longer linker lengths. The human and *E. coli* A-site targets exhibit a conserved G-C, non-canonical U-U, and C-G base-pair before the A-site bulge. The bulge region in human A-site, however, contains a G1408, whereas in *E. coli* A-site is contains an adenine base in this position. The region after the bulge also differs between *E. coli* and human RNA. PYR-NEO conjugates with shorter linkers could make favorable interactions in the *E. coli* rRNA bulge compared to the bulge region in human A-site targets. For optimum *E. coli* A-site specificity, five atoms between NEO III ring and PYR affords sufficient chain length and flexibility for NEO to fit into the bulge region as well as for

pyrene to intercalate or stack. As the linker length increases between NEO and PYR, the entropic costs of the longer linker begins to inhibit simultaneous interactions of pyrene and NEO. It is likely that subsequent pyrene intercalation in conserved regions reduces the selectivity between human and *E. coli* A-sites.

It is known that NEO binds to all rRNA A-site targets with high affinity as is the case reported here, whereas PYR has been shown to be a weak nucleic acid non-specific intercalator. The affinity of NEO to the rRNA A-site determined *via* the F-NEO screening test has been reported previously,²³ with an IC₅₀ value range of 68-114 nM. Most of the PYR-NEO conjugates show much lower affinity towards rRNA A-sites than NEO, except for **13** which has a slightly lower binding affinity compared to NEO. However, all of the PYR-NEO conjugates exhibit slightly higher selectivity to bacterial A-sites than NEO.

Subtle differences in atomic linker-length had a varying impact on the binding affinity and, selectivity to the *E. coli* A-site compared to the human A-site target. The optimum linker length for the highest binding affinity was seven atoms, and this conjugate (**13**) has an almost equivalent binding affinity as NEO to multiple A-site targets. However, deviation to either a lower number linker length or greater number linker length greatly reduced binding affinity to the A-sites. In conjugates with a linker-length below seven atoms, the tethered PYR and a short linker, likely prevent optimal NEO binding to the A-site. In conjugates with atomic lengths above seven atoms, the steric bulk and the entropic cost results in lowering the affinities of the conjugates to the A-site. For most linkers, conjugation of PYR to NEO slightly increases specificity of NEO to bacterial A-sites compared to human cytosolic A-site. Given that NEO binds with exceptionally high affinity to rRNA targets when compared to other aminoglycosides, reduction of affinity could be a means of achieving higher selectivity, as seen from our results.

Screening PYR-NEO conjugates for antibacterial activity and determination of MIC values

The seven PYR-NEO conjugates **10-16** were screened at a single concentration (6.25 μ M) against several bacterial strains to estimate their antimicrobial properties.²⁴ Table 4 lists the strains used in this study and relevant characteristics encompassing Gram-positive and Gram-negative strains of clinical importance. *E. coli* ATCC 25922 and *S. aureus* ATCC 29523 are reference strains used to determine the activity of antibiotics to sensitive strains. Other strains were selected based on their resistance or intermediate sensitivity to NEO. Initial screening showed that all seven compounds exhibited broad spectrum antibacterial activity against Gram-positive strains including methicillin resistant *S. aureus* compared to Gram-negative strains (Table S1).

We have previously demonstrated the length and flexibility of the linker used to conjugate NEO to small molecules plays an important role in RNA binding affinity and antibacterial activity.²⁵⁻²⁶ PYR-NEO conjugates with the shortest linkers that exhibited greater selectivity to the *E. coli* A-site model, had the lowest antibacterial activity (Table S1). That these compounds expressed the lowest percent binding to all A-sites as determined by the F-NEO displacement assay (Table 1) is consistent with their low antibacterial activity. Based on the initial screen for antibacterial activity, compounds **12**, **13**, **15**, and **16** were chosen to identify MIC values for NEO sensitive strains that demonstrated 30-100% growth inhibition and for

all NEO-resistant strains that exhibited any growth inhibition (Table 5). The observation that the *E. Coli* ATCC 25923 NEO-sensitive strain was not susceptible to the PYR-NEO conjugates while the *E. coli* TolC efflux mutant was inhibited by these compounds indicates that active efflux of the PYR-NEO conjugate occurs with Gram-negative bacteria making them more immune to antimicrobial action (Table 5). In contrast, this pattern was not observed between *S. aureus* ATCC 25923 and *S. aureus* NorA (efflux mutant), indicating the permeability barrier imparted by the outer membrane of Gram-negative bacteria is also a factor in uptake and retention of PYR-NEO conjugates. Interestingly, multidrug-resistant *E. coli* H4H had 30% growth inhibition by the compounds with the longest linker lengths **15** (9 atom linker) and **16** (18 atom linker).

Though none of the compounds was more active than NEO with all NEO-sensitive bacterial strains, PYR-NEO compounds **12**, **13**, **15**, and **16** had significantly lower MIC values than NEO for the 4 MRSA NEO-resistant strains with MIC values 4-16 times lower than that of NEO. These MRSA strains in addition to methicillin resistance are also resistant to other aminoglycosides (kanamycin, amikacin, gentamicin, and tobramycin). The observation that PYR-NEO conjugates are more effective than NEO in killing the MRSA strains used here indicates the PYR addition, with an appropriate linker, has allowed us to circumvent the myriad of aminoglycoside resistant mechanisms by these strains. MRSA ATCC A960649 possesses *ant4'* (4'-aminoglycoside nucleotidyltransferase) and *aac6'-aph2''* (6'-aminoglycoside acetyltransferase-2''-aminoglycoside phosphotransferase) AMEs, which make the strain resistant to gentamicin and other aminoglycosides.

AME activity on PYR-NEO conjugates

To determine if PYR-NEO conjugates could resist the action of AMEs, we tested them against a panel of 6 AMEs: AAC(6')-Ie, AAC(3)-IV, AAC(2')-Ic, Eis, APH(2'')-Ia, and APH(3')-Ia. In general all AMEs tested showed reduced reaction rates with conjugates **10-16**, with the exception of **15**, which showed increased activity with APH(2'')-Ia and Eis (Figure 4). Quite remarkably, all of the PYR-NEO conjugates were poor substrates of Eis. This particular AAC modifies multiple amines on aminoglycosides,²⁷ making it very difficult to design aminoglycosides that completely avoid modification by Eis. Our previous work using anthraquinone-NEO conjugates²⁵ showed that even large aromatic modifications cannot always slow this enzyme. All regiospecific AACs showed a reduction in reaction rate with conjugates **10-16** when compared to the rate of the parent NEO. Previous studies by us and others have shown that certain modifications work better for different enzymes. For example, dimerization of NEO with various linkers does not affect the rate of AAC(2')-Ic.²⁶ Conversely, the addition of short peptide chains and aromatic groups seems to slow the rates of all the AMEs tested, as previously reported by us.¹⁰ This observation doesn't just apply to NEO, other modified aminoglycosides have also shown enzyme-selective rate impediments.²⁸⁻³⁰ The knowledge gained by studying compounds **10-16** with AMEs brings us closer to finding a way of preventing drugs from being modified by these enzymes.

In vitro inhibition of translation

A few of the compounds (**10**, **13**, **15**, **16**) tested for antibacterial activity were assayed in a cell-free translation system for prokaryotes (Figure S72). Many of the compounds were

similar or poor inhibitors of protein synthesis at nanomolar concentrations, as compared to the parent aminoglycoside (NEO), with IC_{50} values in the range of 212-1200 nM, compared with NEO (IC_{50} = 139 nM). For example, compound **10**, with the short linker, did not inhibit translation effectively (IC_{50} ~1200 nM), was one of the weakest binders in F-NEO displacement assays (IC_{50} ~300 nM), and exhibited very weak antibacterial activity. These results are consistent with previous reports where some aromatic-NEO conjugates' binding to A-sites correlates well with antibacterial activity and translation inhibition.²⁵ IC_{50} values for compounds **10** and **13** correlate well with the moderate to high antibacterial activities observed against a broad spectrum of bacterial strains. For these compounds, the appropriate linker allows both PYR and NEO binding to inhibit translation. Compounds **15** and **16**, containing longer linkers, are poor inhibitors of translation, yet show Gram-positive antibacterial activities comparable to **13**. An overall weaker translation inhibition activity by most conjugates is consistent with their lower antibacterial activity, when compared to NEO against NEO sensitive strains (Table 5 and Table S2). Moreover, while they are much more active against the NEO resistant strains due to their ability to evade the enzymatic resistance pathways, their lower translation inhibition may be reflected in the MIC values observed for the MRSA strains (6.25-12.5 μ M).

Docking studies

To get possible modes of interaction of PYR-NEO conjugates, we performed docking studies of three PYR-NEO compounds (**11-13**) using AutoDock Vina. AutoDock Vina, an improved version of AutoDock 4, contains a new scoring function providing much precision to ligand-receptor interaction prediction among other advantages. Our initial docking experiments were performed with compound **11** which exhibited best selectivity factor towards bacterial A-site inhibition in comparison to human cytosolic A-site sequence. The docking studies of compound **11** were performed with an oligonucleotide model of prokaryotic A-site reported previously by Puglisi group.³¹ The results from the docking studies of compound **11** showed that NEO occupies nearly the same space as occupied by paromomycin, which is structurally very similar to neomycin, when compared with NMR structure of paromomycin-A-site complex.³¹ The best conformer obtained from this experiment had the binding energy of -9.4 kcal/mol while the lowest energy conformation had the binding energy of -8.0 kcal/mol. Further analysis of docking results with compound **11** revealed that polar and van der Waals interactions are the key driving factors of compound **11**'s interaction with the bacterial A-site where the primary amine on ring IV of NEO displayed hydrogen bonding interactions with the phosphate backbone of A1492 (Figure 5). The secondary amine on Ring IV of NEO makes polar contacts with the adjacent cytosine where the interactions in the groove are further strengthened by polar contacts with the amine connecting NEO with PYR and G1404. The PYR moiety of the molecule accommodates itself towards central part of the groove making π - π interactions with the G1404, U1405 and C1406 bases. In contrast, the docking studies with the human A-site sequence revealed that the best conformation of compound **11** (binding energy of -9.2 kcal/mol) showed interaction of NEO in the groove spanning the upper and lower ends of the lower stem quite similar to the structure of paromomycin bound to eukaryotic A-site as determined using solution NMR previously (Figure 5). The core difference in the bacterial and human A-site structure lies in the change of base at 1408 position (adenine in bacterial

and guanine in human A-site). This single base change in bulge region leads to a reduction in the aminoglycoside affinity to the RNA structure. Further inspection of the binding site provides insights in the differential binding of compound **11**. The PYR moiety in compound **11**, quite dissimilar to its binding to bacterial A-site RNA sequence, orients itself away from the major groove in the vicinity of U1405 and C1406 bases making fewer interactions with the RNA. The presence of the PYR moiety into the vacant space of the groove, drastically reduces van der Waals contacts.

We then performed docking experiments with compound **13** which afforded the best inhibitory concentration (IC₅₀) with the A-site RNA sequence. Here again, the NEO rings make several polar interactions centered in the groove while PYR protrudes away from the groove (Figure S71). Finally, we performed docking experiments with compound **12**, which had an intermediate selectivity factor (6.76). The docking results with **12** also show that polar interactions of the neomycin moiety and the interactions with the pyrene moiety play important roles in conferring extra binding of the PYR-NEO conjugates. The lowest energy conformations of both bacterial and human A-site complexes are shown in the supporting information (Figure S71). Additional docked poses (second and third lowest energy conformations) of compound **11** showed that both neomycin and pyrene moieties occupy same region of the major groove with variations in the ring conformations of neomycin or flipping of positions of the two units (Figure S 71C).

Toxicity screen of select pyrene-neomycin conjugates in *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* has been used to evaluate the potential toxicity of novel drugs, toxicity to mitochondria and efficacy in clearing multiple-drug resistant *Staphylococcus aureus* (MRSA) and other bacteria. We evaluated the toxicity of two pyrene-neomycin conjugates in the *C. elegans* animal model. The pyrene-neomycin conjugates DPA543 and DPA544 were chosen based on their overall higher binding affinity to the bacterial A site rRNA model, activity against MRSA and low aminoglycoside-modifying enzyme activity relative to neomycin.

Little significant difference in toxicity was observed between neomycin and the PYR-NEO conjugates within the concentration range of 6.25 – 50 μ M with a decrease in formazan production (an indicator of the nematode larvae viability) ranging from ~ 50 – 75%. There was no significant difference in toxicity of NEO and the untreated control as compared to the PYR-NEO conjugates at the lowest concentrations used (3.13 – 1.56 μ M). The significant reduction in larvae viability at the lowest concentration of the PYR-NEO conjugates suggests some contribution by the PYR moiety itself. Previous studies have shown that polycyclic aromatic hydrocarbons reduce the activity and reproductive capacity of adult nematodes and kill L1-stage larvae at concentrations as low as 2.5 μ g/mL.⁵⁰

Conclusion

The global spread of multidrug-resistant bacterial strains drives the imperative to find novel antimicrobials that combat this serious public health problem. We show that the conjugation of NEO *via* the 5''-OH position with PYR and an appropriate linker length leads to higherspecificity of the PYR-NEO to *E. coli* A-site rRNA than NEO alone and demonstrates

the utility of simple and efficient conjugation of readily available nucleic acid binders. The conjugation of pyrene to NEO through the 5'-OH position significantly resists action from AMEs on NEO. PYR-NEO conjugates have broad spectrum antibacterial activity towards Gram-positive strains, but not with Gram-negative bacteria. Use of drugs that compromise the outer membrane of Gram-negative bacteria could sensitize them to antibacterial action of the PYR-NEO conjugates.³² Significant growth inhibition was observed with *S. aureus*, *B. anthracis*, *M. smegmatis*, and *L. monocytogenes*. That some PYR-NEO compounds were more effective than NEO in killing NEO-resistant *S. aureus* strains and a multidrug-resistant *E. coli* strain indicates the action of AMEs have been successfully evaded. Further work to optimize the linker length and composition leading to improved translation inhibition and antibacterial effects is underway and will be reported in due course.

Experimental section

General methods

Chemicals for the preparation of PYR intermediates were purchased from Sigma-Aldrich and used as received. NEO was purchased from MP Biomedicals (Solon, OH) as sulfate salt. Di-*tert*-butyl dicarbonate was purchased from Advanced Chem Tech (Louisville, KY). All solvents were purchased from VWR (Atlanta, GA). Silica gel was purchased from Sorbent (Atlanta, GA). ¹H NMR and ¹³C NMR were recorded on Bruker Avance (300/500 MHz) spectrometers. Chemical shifts are given in ppm and are referenced to residual solvent peaks (¹H and ¹³C NMR). Mass (MALDI-TOF) spectra were collected using a Bruker Microflex mass spectrometer. Ultraviolet (UV-Vis) spectra were collected on a Varian (Walnut Creek, CA) Cary 100 Bio UV-Vis spectrophotometer equipped with a thermoelectrically controlled 12-cell holder. HPLC analysis of all PYR-NEO conjugates were performed on a HP1100 series analytical instrument. All conjugates exhibited a final purity of >95%.

Synthesis

The synthesis of the PYR-NEO conjugates **10-16** (Figure 1) is summarized in Scheme 1. All conjugates were purified using column chromatography and were deprotected with trifluoroacetic acid and lyophilized with a Labconco freeze dryer system prior to use. NMR characterization was performed on either a Bruker 500 MHz or a JEOL 500 MHz NMR spectrometer. All mass spectra were obtained using a Bruker MALDI mass spectrometer. All UV-Vis data were obtained using a Cary 100 UV-Vis spectrophotometer.

Synthesis of 4-(2,3-dihdropyren-2-yl)-N-(6-isothiocyanatohexyl)butanamide (compound 7)

Pyrene succinimidyl ester intermediates (**4-6**) were prepared according to literature protocol.³³ Into a clean and dry 100 mL round bottom flask, 1,6-hexanediamine (5 g, 43 mmol) was dissolved in 10 mL of anhydrous pyridine. The reaction flask was purged with argon and covered with aluminum foil. In a separate vial, a solution of pyrene succinimidyl ester **6** (0.200 g, 0.519 mmol) in 10 mL of dry pyridine was prepared. The solution containing compound **6** was added dropwise to the reaction flask over 2 h and subsequently allowed to stir overnight. The solvent was then dried under reduced pressure. The residue was purified *via* column chromatography on a silica gel column (CH₂Cl₂:MeOH, 9:1 v/v) to afford product **7a** as a waxy yellow solid (0.246 g, 92%): *R*_f = 0.38 in 9:1/CH₂Cl₂:MeOH; ¹H NMR

(500 MHz, DMSO- d_6) δ 8.38 (d, J = 9.2 Hz, 1H), 8.26 (d, J = 7.7 Hz, 1H), 8.23-8.17 (m, 2H), 8.16-8.09 (m, 2H), 8.09-8.00 (m, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.83 (s, 1H), 3.31 (d, J = 7.2 Hz, 2H), 3.09-3.04 (m, 2H), 2.22 (t, J = 6.7 Hz, 2H), 2.00 (d, J = 6.0 Hz, 2H), 1.86 (s, 1H), 1.68 (s, 1H), 1.50-1.35 (m, 3H), 1.34-1.23 (m, 5H), 1.17 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 171.81, 136.75, 131.06, 130.60, 129.47, 128.32, 127.69, 127.63, 127.38, 126.67, 126.31, 125.11, 124.97, 124.41, 124.32, 123.66, 50.70, 38.62, 35.21, 32.44, 30.88, 30.63, 29.41, 28.90, 27.80, 26.98, 26.58, 26.31; MS (MALDI-TOF) m/z Calcd for $\text{C}_{26}\text{H}_{31}\text{N}_2\text{O}$: 387.25 (M+H) $^+$, Found: 387.33.

Pyrene hexamethylene amine **7a** (0.1 g, 0.259 mmol) was dissolved in dry pyridine (20 mL) in a dry 100 mL round bottom flask and 1,1'-thiocarbonyldi-2(1H)-pyridone (TCDP) (0.24 g, 1.04 mmol) was then added to this solution. The flask was wrapped in aluminum foil and allowed to stir for 5 h. The solvent was dried under reduced pressure and the reaction mixture was purified *via* column chromatography to afford the product (compound **7**) as a white solid: R_f = 0.51 in 2:1/hexanes:EtOAc; ^1H NMR (500 MHz, DMSO- d_6) δ 8.38 (d, J = 9.3 Hz, 1H), 8.27 (t, J = 7.8 Hz, 2H), 8.22 (dd, J = 8.5, 4.3 Hz, 2H), 8.13 (d, J = 3.4 Hz, 1H), 8.06 (t, J = 7.6 Hz, 1H), 7.94 (d, J = 7.8 Hz, 1H), 7.82 (t, J = 5.5 Hz, 1H), 3.99 (d, J = 4.0 Hz, 1H), 3.90 (d, J = 12.4 Hz, 1H), 3.63 (t, J = 6.5 Hz, 2H), 3.36 – 3.27 (m, 2H), 3.06 (m, 2H), 2.22 (t, J = 7.2 Hz, 2H), 2.10-1.96 (m, 2H), 1.63-1.58 (m, 2H), 1.48-1.37 (m, 3H), 1.35-1.28 (m, 3H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 172.14, 137.04, 131.35, 130.90, 129.77, 128.62, 128.00, 127.93, 127.68, 126.97, 126.61, 125.41, 125.27, 124.71, 124.62, 123.97, 53.07, 45.13, 38.79, 35.48, 32.73, 29.64, 29.48, 28.06, 26.18, 26.09; IR (neat, cm^{-1}) 3090 (N-H), 2973, 2927 (C-H), 2173, 2050 (NCS), 750, 710 (aromatic C-H); MS (MALDI-TOF) m/z Calcd for $\text{C}_{27}\text{H}_{28}\text{N}_2\text{OS}$: 428.19 (M) $^+$, Found: 428.29.

General procedure for the synthesis of PYR-NEO conjugates (10-16)

The pertinent NEO-amine intermediates were synthesized using previously established protocols.^{16, 18, 25} To a solution of the corresponding *N*-Boc-protected NEO amine in dry pyridine, a solution of pyrene succinimidyl ester was added followed by addition of a catalytic amount of 4-dimethylaminopyridine (DMAP). The flask was purged with argon and covered in aluminum foil. The reaction mixture was allowed to stir for 18 h and monitored *via* TLC, to assess the formation of the product. Pyridine was then dried under reduced pressure. The crude reaction mixture was purified by column chromatography using CH_2Cl_2 :MeOH as eluent to afford the desired Boc-protected conjugates as white powders (59-82%). The conjugates were subsequently dissolved in CH_2Cl_2 and the amines were de-protected *via* the addition of trifluoroacetic acid. The crude product was dissolved in deionized water and extracted with CH_2Cl_2 . Products were then freeze-dried to afford the final conjugates as white powders in 81-88% yield.

N-Boc-protected compound 15 (Protected DPA 503)

(13 mg, 67%); R_f = 0.55 in 9:1/ CH_2Cl_2 :MeOH; ^1H NMR (300 MHz, Acetone- d_6) δ 8.46 (dd, J = 9.3, 1.9 Hz, 1H), 8.29-8.24 (m, 1H), 8.29-8.20 (m, 2H), 8.18 (dd, J = 4.6, 2.6 Hz, 1H), 8.13-8.07 (m, 2H), 8.08-7.99 (m, 1H), 7.97 (dd, J = 7.8, 1.9 Hz, 1H), 6.44-6.15 (m, 3H), 6.10-5.88 (m, 2H), 5.16 (d, J = 3.1 Hz, 1H), 4.96-4.93 (m, 1H), 4.77 (s, 1H), 4.47-4.18 (m, 4H), 4.18 (s, 2H), 4.09-4.00 (m, 2H), 3.89-3.76 (m, 4H), 3.52-3.36 (m, 5H), 3.34-3.14

(m, 3H), 2.55-2.44 (m, 2H), 2.19-2.08 (m, 2H), 1.44-1.40 (m, 55H); ^{13}C NMR (75 MHz, Acetone- d_6) δ 174.65, 158.23, 157.49, 157.02, 156.75, 156.26, 137.40, 132.37, 131.90, 130.86, 129.59, 128.42, 128.40, 128.17, 127.49, 126.88, 125.84, 125.79, 125.67, 124.42, 111.03, 101.05, 99.89, 87.92, 83.81, 90.97, 79.81, 79.64, 79.21, 79.06, 78.88, 75.71, 73.73, 73.05, 72.02, 71.05, 63.46, 57.11, 53.33, 42.66, 41.03, 35.82, 33.82, 33.32, 28.84, 28.76, 28.70, 28.64, 28.60, 27.88; MS (MALDI-TOF) m/z Calcd for: $\text{C}_{75}\text{H}_{113}\text{N}_7\text{O}_{25}\text{SNa}$: 1567.803 ($\text{M}+\text{Na}$) $^+$, Found: 1567.23.

N-Boc-protected compound 10 (Protected DPA 541)

(9 mg, 78%); R_f = 0.58 in 9:1/ CH_2Cl_2 :MeOH; ^1H NMR (300 MHz, Acetone- d_6) δ 8.42 (d, J = 9.1 Hz, 1H), 8.26 (s, 1H), 8.23 (d, J = 2.8 Hz, 1H), 8.20 (s, 1H), 8.18 (d, J = 2.3 Hz, 1H), 8.08 (d, J = 2.2 Hz, 2H), 8.02 (d, J = 7.4 Hz, 1H), 7.95 (s, 1H), 7.59 (s, 1H), 6.45 (s, 1H), 6.29 (s, 1H), 6.19 (d, J = 9.4 Hz, 1H), 6.07 (d, J = 10.4 Hz, 2H), 5.84 (s, 1H), 5.14-5.10 (m, 2H), 5.00 (d, J = 1.8 Hz, 1H), 4.22 (d, J = 5.4 Hz, 4H), 4.02 (s, 2H), 3.90 (d, J = 7.4 Hz, 1H), 3.81-3.75 (m, 5H), 3.56 (s, 3H), 3.41 (d, J = 9.8 Hz, 6H), 3.26-3.13 (m, 4H), 2.78 (d, J = 7.2 Hz, 5H), 2.39 (t, J = 7.2 Hz, 3H), 2.20-2.09 (m, 3H), 1.41-1.37 (m, 55H); ^{13}C NMR (75 MHz, Acetone- d_6) δ 174.65, 158.22, 157.60, 157.48, 157.2, 156.26, 137.40, 132.37, 131.90, 130.86, 129.59, 128.42, 128.40, 128.18, 128.17, 127.49, 126.88, 125.84, 125.79, 125.67, 124.42, 110.97, 101.06, 99.88, 87.92, 83.83, 79.81, 79.64, 79.20, 79.06, 78.86, 75.71, 73.74, 73.06, 72.03, 71.04, 68.27, 67.59, 63.41, 57.11, 54.95, 53.32, 51.96, 51.81, 42.60, 41.03, 33.83, 33.32, 28.84, 28.76, 28.70, 28.64, 28.60, 27.88; MS (MALDI-TOF) m/z Calcd for $\text{C}_{71}\text{H}_{105}\text{N}_7\text{O}_{25}\text{Na}$: 1479.64 ($\text{M}+\text{Na}$) $^+$, Found: 1479.68.

N-Boc-protected compound 12 (Protected DPA 542)

(6 mg, 82%); R_f = 0.60 in 9:1/ CH_2Cl_2 :MeOH; ^1H NMR (500 MHz, Acetone- d_6) δ 8.48 (d, J = 9.3 Hz, 1H), 8.27 (t, J = 6.9 Hz, 2H), 8.23 (dd, J = 8.5, 4.3 Hz, 2H), 8.16-8.10 (m, 2H), 8.06 (t, J = 7.6 Hz, 1H), 7.99 (d, J = 7.8 Hz, 1H), 6.44-6.29 (m, 2H), 6.27 (d, J = 5.7 Hz, 1H), 6.15 (d, J = 8.0 Hz, 1H), 6.04 (t, J = 10.3 Hz, 1H), 5.97-5.89 (m, 1H), 5.18 (d, J = 3.1 Hz, 1H), 4.98-4.96 (m, 1H), 4.78 (s, 1H), 4.41-4.28 (m, 3H), 4.21-4.18 (m, 2H), 4.11-4.03 (m, 2H), 3.91-3.83 (m, 2H), 3.83-3.78 (m, 2H), 3.59-3.52 (m, 2H), 3.52-3.43 (m, 5H), 3.24 (dd, J = 13.5, 6.3 Hz, 2H), 2.53 (t, J = 7.2 Hz, 2H), 2.20-2.13 (m, 2H), 2.10-2.04 (m, 3H), 1.50-1.40 (m, 54H); ^{13}C NMR (125 MHz, Acetone- d_6) δ 171.1, 157.0, 156.1, 155.1, 131.4, 131.0, 130.5, 129.6, 128.8, 127.6, 127.4, 127.0, 126.0, 125.0, 124.9, 124.9, 124.8, 124.6, 123.9, 110.92, 101.0, 99.9, 82.2, 81.7, 79.0, 78.8, 78.3, 78.0, 77.9, 73.1, 72.6, 72.1, 70.1, 56.0, 54.0, 52.5, 50.1, 41.1, 40.0, 39.2, 38.0, 37.4, 34.1, 31.3, 29.4, 29.3, 29.3, 29.2, 29.1, 29.0, 28.9, 28.7, 28.5, 28.4, 27.9, 27.8, 27.7, 27.6, 27.1, 25.1, 25.1, 22.8; MS (MALDI-TOF) m/z Calcd for $\text{C}_{73}\text{H}_{109}\text{N}_7\text{O}_{25}\text{Na}$: 1506.737 ($\text{M}+\text{Na}$) $^+$, Found: 1506.341.

N-Boc-protected compound 13 (Protected DPA 543)

(19 mg, 77%); R_f = 0.52 in 9:1/ CH_2Cl_2 :MeOH; ^1H NMR (300 MHz, Acetone- d_6) δ 8.47 (d, J = 9.3 Hz, 1H), 8.37-8.29 (m, 3H), 8.27 (d, J = 1.4 Hz, 1H), 8.18 (s, 2H), 8.16-8.06 (m, 2H), 7.67 (s, 1H), 6.43 (s, 1H), 6.32 (s, 1H), 6.24 (d, J = 9.5 Hz, 1H), 6.15 (d, J = 9.5 Hz, 2H), 5.92 (d, J = 10.2 Hz, 1H), 5.25-5.12 (m, 2H), 5.10-5.04 (m, 1H), 4.77 (d, J = 3.3 Hz, 1H), 4.56-4.37 (m, 3H), 4.37-4.14 (m, 4H), 4.14-3.99 (m, 1H), 3.98-3.75 (m, 4H), 3.74-3.36 (m, 10H), 3.35-3.02 (m, 4H), 2.79 (t, J = 6.9 Hz, 2H), 1.65-1.21 (m, 54H); ^{13}C NMR (126 MHz,

Aceton- d_6) δ 172.01, 158.42, 157.57, 157.02, 156.68, 156.27, 132.36, 131.94, 131.53, 130.57, 129.77, 128.59, 128.43, 127.96, 127.01, 126.05, 126.02, 125.92, 125.83, 125.58, 124.91, 110.99, 101.96, 100.11, 87.57, 83.18, 82.69, 81.49, 79.95, 79.81, 79.27, 79.04, 78.88, 75.50, 75.34, 74.12, 73.64, 73.16, 72.54, 71.09, 68.26, 56.97, 54.95, 53.51, 52.06, 42.75, 41.77, 40.98, 40.20, 35.91, 34.95, 34.24, 32.29, 28.93, 28.90, 28.81, 28.78, 28.69, 28.63, 26.12; MS (MALDI-TOF) m/z Calcd for $C_{73}H_{109}N_7O_{25}SNa$: 1538.709 (M+Na) $^+$, Found: 1538.921.

N-Boc-protected compound 14 (Protected DPA 544)

(13 mg, 59%); R_f = 0.55 in 9:1/ CH_2Cl_2 :MeOH; 1H NMR (300 MHz, Acetone- d_6) δ 8.48 (d, J = 9.3 Hz, 1H), 8.29-8.26 (m, 1H), 8.24 (dd, J = 4.7, 2.6 Hz, 2H), 8.20 (d, J = 3.6 Hz, 1H), 8.16-8.08 (m, 2H), 8.07-8.02 (m, 1H), 7.98 (d, J = 7.8 Hz, 1H), 6.50-6.10 (m, 3H), 6.08-5.86 (m, 2H), 5.17 (s, 1H), 4.96 (s, 1H), 4.78 (s, 1H), 4.39-4.32 (m, 2H), 3.91-3.80 (m, 5H), 3.65-3.58 (m, 3H), 3.56-3.49 (m, 2H), 3.483.40 (m, 4H), 3.22 (d, J = 7.0 Hz, 3H), 2.97 (s, 2H), 2.52 (t, J = 7.2 Hz, 2H), 2.29-2.12 (m, 2H), 1.68-1.06 (m, 54H); ^{13}C NMR (126 MHz, Acetone- d_6) δ 172.01, 158.42, 157.57, 157.02, 156.68, 156.27, 132.36, 131.94, 131.53, 130.57, 129.77, 128.59, 128.43, 127.96, 127.01, 126.05, 126.02, 125.92, 125.83, 125.58, 124.91, 110.99, 101.96, 100.11, 87.57, 83.18, 82.69, 81.49, 79.95, 79.81, 79.27, 79.04, 78.88, 75.50, 75.34, 74.12, 73.64, 73.16, 72.55, 72.54, 71.09, 68.26, 56.97, 54.95, 53.51, 52.06, 51.71, 42.75, 41.77, 40.98, 40.20, 39.45, 35.91, 34.95, 34.24, 32.29, 28.93, 28.90, 28.81, 28.78, 28.69, 28.63, 26.12; MS (MALDI-TOF) m/z Calcd for $C_{74}H_{111}N_7O_{25}SNa$: 1553.78 (M+Na) $^+$, Found: 1554.23.

N-Boc-protected compound 16 (Protected DPA 547)

(13 mg, 72%); R_f = 0.48 in 9:1/ CH_2Cl_2 :MeOH; 1H NMR (300 MHz, Acetone- d_6) δ 8.46 (d, J = 9.3 Hz, 1H), 8.32-8.24 (m, 3H), 8.21 (s, 1H), 8.13 (d, J = 1.6 Hz, 2H), 8.07 (d, J = 7.6 Hz, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.35-7.18 (m, 2H), 6.41 (s, 1H), 6.29 (s, 1H), 6.20 (d, J = 9.3 Hz, 1H), 6.10 (d, J = 9.4 Hz, 2H), 5.92 (s, 1H), 5.22 (s, 1H), 5.10 (s, 1H), 5.02 (s, 1H), 4.79 (s, 1H), 4.23 (d, J = 6.9 Hz, 5H), 4.07 (s, 1H), 3.94 (s, 1H), 3.82 (d, J = 9.9 Hz, 3H), 3.60 (s, 5H), 3.48-3.36 (m, 4H), 3.25 (d, J = 6.4 Hz, 5H), 2.37 (t, J = 7.1 Hz, 2H), 2.27-2.13 (m, 2H), 1.69-1.30 (m, 72H), 0.89 (s, 2H); ^{13}C NMR (75 MHz, Acetone- d_6) δ 174.64, 158.24, 157.58, 157.47, 157.01, 156.25, 137.39, 132.36, 131.89, 130.85, 129.58, 128.41, 128.39, 128.17, 127.48, 126.87, 125.83, 125.78, 125.66, 124.41, 110.96, 101.05, 99.87, 87.92, 83.83, 79.81, 79.64, 79.20, 79.06, 78.86, 75.71, 73.74, 73.05, 72.03, 71.06, 68.27, 67.59, 57.11, 54.94, 53.32, 51.97, 42.72, 42.60, 41.03, 33.83, 33.32, 28.84, 28.76, 28.70, 28.64, 28.60, 27.88; MS (MALDI-TOF) m/z Calcd for $C_{82}H_{127}N_9O_{25}S_2Na$: 1726.07 (M+Na) $^+$, Found: 1726.19.

N-Boc-protected compound 11 (Protected DPA 548)

(11 mg, 71%); R_f = 0.60 in 9:1/ CH_2Cl_2 :MeOH; 1H NMR (500 MHz, Acetone- d_6) δ 8.55 (d, J = 9.1 Hz, 1H), 8.30-8.24 (m, 2H), 8.22 (d, J = 7.3 Hz, 1H), 8.16-8.08 (m, 2H), 8.08-8.02 (m, 1H), 8.01 (d, J = 7.9 Hz, 2H), 7.21-7.15 (m, 1H), 6.44 (d, J = 9.8 Hz, 1H), 6.20 (s, 1H), 6.05 (d, J = 10.1 Hz, 1H), 5.92 (s, 1H), 5.19 (s, 1H), 5.14 (d, J = 4.7 Hz, 1H), 4.97-4.93 (m, 1H), 4.75 (d, J = 3.6 Hz, 1H), 4.41-4.22 (m, 2H), 4.22-4.10 (m, 3H), 4.08-3.92 (m, 2H), 3.90-3.74 (m, 3H), 3.74-3.59 (m, 4H), 3.53 (s, 1H), 3.51-3.41 (m, 4H), 3.40-3.27 (m, 3H), 2.58-2.50

(m, 2H), 2.23-2.14 (m, 2H), 1.59-1.12 (m, 54H), 0.93-0.85 (m, 2H); ^{13}C NMR (125 MHz, Acetone- d_6) δ 171.6, 157.0, 156.6, 156.0, 155.6, 155.3, 131.4, 130.9, 130.5, 129.6, 128.8, 127.6, 127.4, 127.0, 126.0, 125.0, 124.9, 124.8, 124.8, 124.6, 123.9, 110.90, 101.01, 99.1, 82.1, 81.7, 79.1, 78.6, 78.3, 78.1, 77.9, 73.1, 72.7, 72.1, 70.1, 56.0, 54.0, 52.5, 40.8, 40.0, 39.2, 34.1, 31.3, 29.4, 29.2, 29.2, 29.1, 29.1, 29.0, 28.9, 28.7, 28.5, 28.4, 27.9, 27.8, 27.7, 25.1, 25.0, 24.8, 23.4; MS (MALDI-TOF) m/z Calcd for $\text{C}_{72}\text{H}_{107}\text{N}_7\text{O}_{25}\text{Na}$: 1493.66 (M+Na) $^+$, Found: 1494.12.

Compound 15 (DPA 503)

(6 mg, 86%); ^1H NMR (300 MHz, D_2O) δ 8.15 (dd, J = 8.0, 4.2 Hz, 3H), 8.11-7.90 (m, 5H), 7.83 (d, J = 7.6 Hz, 1H), 5.91 (d, J = 4.0 Hz, 1H), 5.29 (d, J = 3.9 Hz, 1H), 4.94 (dd, J = 3.2, 1.7 Hz, 1H), 4.22-4.17 (m, 2H), 4.14-4.03 (m, 2H), 4.02-3.86 (m, 2H), 3.85-3.73 (m, 3H), 3.70-3.56 (m, 2H), 3.39 (d, J = 12.7 Hz, 3H), 3.25 (s, 3H), 3.17 (d, J = 6.3 Hz, 2H), 3.04-2.80 (m, 4H), 2.77-2.66 (m, 1H), 2.66-2.24 (m, 7H), 2.17 (d, J = 4.9 Hz, 1H), 2.07 (dd, J = 13.7, 6.5 Hz, 3H), 1.91-1.78 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 178.38, 159.61, 147.14, 142.37, 135.21, 129.56, 129.48, 129.43, 128.04, 127.26, 126.91, 123.12, 122.43, 118.51, 114.93, 110.15, 95.07, 84.74, 81.19, 74.83, 73.30, 72.39, 70.99, 70.32, 69.51, 67.95, 67.66, 67.19, 60.20, 57.23, 53.65, 50.84, 49.96, 48.50, 40.41, 35.98, 33.37, 28.43, 27.92, 21.31, 19.55, 17.74; MS (MALDI-TOF) m/z Calcd for $\text{C}_{45}\text{H}_{65}\text{N}_7\text{O}_{13}\text{SNa}$: 966.43 (M+Na) $^+$, Found: 966.901; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{45}\text{H}_{65}\text{N}_2\text{O}_{13}\text{SNa}$: 966.4259 (M+Na) $^+$, Found: 966.4222; UV (0.008 mM in DI water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 7.22 min, purity 98%.

Compound 10 (DPA 541)

(5 mg, 82%); ^1H NMR (300 MHz, D_2O) δ 8.35 (s, 1H), 8.33 (s, 2H), 8.30 (d, J = 4.6 Hz, 1H), 8.20 (s, 3H), 8.15 (d, J = 7.3 Hz, 1H), 8.10 (s, 1H), 5.98 (d, J = 4.1 Hz, 1H), 5.26 (d, J = 4.2 Hz, 1H), 4.92 (s, 1H), 4.38 (s, 1H), 4.04 (s, 2H), 3.97 (s, 2H), 3.94 (s, 2H), 3.72 (s, 2H), 3.51 (s, 2H), 3.45 (s, 2H), 3.25 (s, 2H), 3.17 (s, 2H), 2.74-2.69 (m, 2H), 2.67 (s, 2H), 2.52 (d, J = 5.5 Hz, 3H), 1.86 (d, J = 12.0 Hz, 2H), 1.29-1.22 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 181.73, 161.52, 148.99, 139.04, 137.12, 132.46, 131.48, 128.84, 124.29, 120.87, 120.44, 119.75, 112.14, 109.47, 97.09, 86.73, 83.15, 76.82, 75.23, 74.35, 72.96, 72.31, 71.46, 69.90, 69.61, 69.12, 62.09, 59.20, 55.58, 52.79, 51.90, 50.44, 42.34, 38.16, 36.38, 30.49, 29.87, 23.71, 21.42; MS (MALDI-TOF) m/z Calcd for $\text{C}_{41}\text{H}_{57}\text{N}_7\text{O}_{13}\text{Na}$: 878.39 (M+Na) $^+$, Found: 878.213; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{41}\text{H}_{58}\text{N}_7\text{O}_{13}$: 856.4093 (M+H) $^+$, Found: 856.4068; UV (0.008 mM in DI water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 6.02 min, purity 97%.

Compound 12 (DPA 542)

(5 mg, 81%); ^1H NMR (300 MHz, D_2O) δ 8.34 (s, 3H), 8.23 (dd, J = 14.8, 8.7 Hz, 3H), 8.03 (dd, J = 8.9, 7.0 Hz, 1H), 7.81 (t, J = 7.7 Hz, 1H), 7.71 (d, J = 6.1 Hz, 1H), 5.93 (d, J = 3.9 Hz, 1H), 5.34 (d, J = 2.2 Hz, 1H), 5.22 (s, 1H), 4.47 (dd, J = 7.0, 4.5 Hz, 1H), 4.41-4.37 (m, 1H), 4.31 – 4.24 (m, 2H), 4.17 (d, J = 3.3 Hz, 1H), 3.93 (s, 1H), 3.90 (s, 1H), 3.87 (s, 1H), 3.84 (d, J = 3.1 Hz, 1H), 3.75 (d, J = 2.6 Hz, 1H), 3.70 (d, J = 5.2 Hz, 1H), 3.64 (dd, J = 11.2, 7.5 Hz, 2H), 3.50 (d, J = 2.5 Hz, 1H), 3.44-3.40 (m, 2H), 3.39-3.34 (m, 3H), 3.30 (dd, J = 7.5, 3.6 Hz, 3H), 3.25 (dd, J = 7.3, 4.5 Hz, 2H), 3.16 (dd, J = 13.6, 7.7 Hz, 2H), 2.352-2.3

(m, 2H), 2.13 (t, $J = 7.1$ Hz, 2H), 1.97-1.88 (m, 2H), 1.62-1.52 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 179.77, 159.55, 147.02, 137.07, 135.15, 130.49, 129.51, 126.87, 122.32, 118.90, 118.47, 117.78, 110.17, 107.50, 95.12, 84.76, 81.18, 74.85, 73.26, 72.38, 70.99, 70.34, 69.49, 67.93, 67.65, 67.15, 60.12, 57.23, 53.61, 50.82, 49.93, 48.46, 40.37, 36.19, 34.41, 28.52, 27.91, 21.75, 19.45; MS (MALDI-TOF) m/z Calcd for $\text{C}_{43}\text{H}_{61}\text{N}_7\text{O}_{13}\text{Na}$: 906.987 ($\text{M} + \text{Na}$) $^+$, Found: 907.099; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{43}\text{H}_{62}\text{N}_7\text{O}_{13}$: 884.4406 ($\text{M} + \text{H}$) $^+$, Found: 884.4394; UV (water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 6.72 min, purity 96%.

Compound 13 (DPA 543)

(6 mg, 88%); ^1H NMR (300 MHz, D_2O) δ 8.34 (d, $J = 6.9$ Hz, 4H), 8.30 (d, $J = 4.6$ Hz, 1H), 8.20 (s, 2H), 8.16 (d, $J = 7.3$ Hz, 1H), 8.08 (d, $J = 7.9$ Hz, 1H), 5.98 (d, $J = 4.1$ Hz, 1H), 5.26 (d, $J = 4.2$ Hz, 1H), 4.92 (s, 1H), 4.37 (d, $J = 6.2$ Hz, 2H), 4.04 (s, 1H), 3.97 (s, 2H), 3.94 (s, 3H), 3.87 (d, $J = 9.2$ Hz, 1H), 3.72 (s, 1H), 3.55-3.41 (m, 10H), 3.29 (s, 2H), 3.25 (s, 2H), 2.73 (dd, $J = 11.2, 6.1$ Hz, 3H), 2.52 (d, $J = 5.5$ Hz, 2H), 1.86 (d, $J = 12.0$ Hz, 1H), 1.25 (d, $J = 8.6$ Hz, 2H); ^{13}C NMR (75 MHz, D_2O) δ 178.93, 158.71, 146.18, 136.23, 134.31, 129.65, 128.67, 126.03, 121.48, 118.06, 117.63, 116.94, 109.33, 106.66, 94.28, 83.92, 80.34, 74.01, 72.42, 71.54, 70.15, 69.50, 68.65, 67.09, 66.81, 66.31, 59.28, 56.39, 52.77, 49.98, 49.09, 47.62, 39.53, 35.35, 33.57, 27.68, 27.07, 20.91, 18.61; MS (MALDI-TOF) m/z Calcd for $\text{C}_{43}\text{H}_{61}\text{N}_7\text{O}_{13}\text{SNa}$: 938.39 ($\text{M} + \text{Na}$) $^+$, Found: 938.387; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{43}\text{H}_{61}\text{N}_7\text{O}_{13}\text{SNa}$: 938.3946 ($\text{M} + \text{Na}$) $^+$, Found: 938.3996; UV (0.008 mM in DI water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 7.19 min, purity 96%.

Compound 14 (DPA 544)

(7 mg, 81%); ^1H NMR (300 MHz, D_2O) δ 8.17 (s, 1H), 8.13 (d, $J = 3.5$ Hz, 2H), 8.06 (s, 1H), 8.03 (d, $J = 4.0$ Hz, 1H), 7.99 – 7.96 (m, 3H), 7.82 (d, $J = 7.8$ Hz, 1H), 5.90 (d, $J = 4.0$ Hz, 1H), 5.29 (d, $J = 3.9$ Hz, 1H), 4.20 (d, $J = 4.6$ Hz, 2H), 4.10 (d, $J = 4.8$ Hz, 1H), 4.08-4.02 (m, 1H), 4.00 (d, $J = 9.2$ Hz, 1H), 3.91 (dd, $J = 10.7, 8.8$ Hz, 2H), 3.82 (d, $J = 4.4$ Hz, 1H), 3.77 (s, 1H), 3.67 (s, 1H), 3.65-3.58 (m, 2H), 3.41 (s, 2H), 3.26 (s, 3H), 3.09 (d, $J = 5.0$ Hz, 1H), 3.01 (d, $J = 3.8$ Hz, 1H), 2.86 (s, 1H), 2.75-2.66 (m, 2H), 2.56 (d, $J = 5.5$ Hz, 1H), 2.51 (d, $J = 6.6$ Hz, 1H), 2.33 (d, $J = 6.9$ Hz, 3H), 2.17 (d, $J = 5.0$ Hz, 1H), 2.13-2.07 (m, 2H), 2.05 (d, $J = 6.8$ Hz, 1H), 1.85 (d, $J = 12.6$ Hz, 2H); ^{13}C NMR (75 MHz, D_2O) δ 178.41, 159.61, 147.14, 142.36, 137.03, 135.21, 129.56, 129.43, 128.05, 127.26, 126.91, 123.12, 122.43, 118.51, 114.94, 110.15, 95.12, 84.74, 81.19, 74.83, 73.30, 72.39, 70.99, 70.32, 69.52, 67.95, 67.67, 67.19, 60.21, 57.23, 53.65, 50.85, 49.95, 48.50, 40.41, 35.99, 33.39, 28.43, 27.92, 21.32, 19.55, 17.74; MS (MALDI-TOF) m/z Calcd for $\text{C}_{44}\text{H}_{63}\text{N}_7\text{O}_{13}\text{SNa}$: 953.074 ($\text{M} + \text{Na}$) $^+$, Found: 954.773; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{44}\text{H}_{64}\text{N}_7\text{O}_{13}\text{S}$: 930.4283 ($\text{M} + \text{H}$) $^+$, Found: 930.4298; UV (0.007 mM in DI water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 7.19 min, purity 96%.

Compound 16 (DPA 547)

(6 mg, 82%); ^1H NMR (300 MHz, D_2O) δ 8.37 (d, $J = 8.5$ Hz, 1H), 8.30 (d, $J = 9.0$ Hz, 1H), 8.18-8.07 (m, 1H), 8.03 (d, $J = 8.2$ Hz, 1H), 7.91 (t, $J = 7.6$ Hz, 1H), 7.84 (dd, $J = 10.5, 7.4$ Hz, 2H), 7.74 (t, $J = 7.9$ Hz, 1H), 7.61 (t, $J = 7.8$ Hz, 1H), 6.09 (d, $J = 3.8$ Hz, 1H), 5.45 (d, J

= 2.1 Hz, 1H), 5.31 (d, J = 2.0 Hz, 1H), 4.91 (t, J = 7.4 Hz, 3H), 4.59-4.53 (m, 1H), 4.48 (dd, J = 4.6, 2.1 Hz, 1H), 4.40-4.31 (m, 1H), 4.23 (dd, J = 12.4, 6.5 Hz, 4H), 4.05 (d, J = 17.0 Hz, 3H), 4.01 (d, J = 4.0 Hz, 3H), 3.99-3.92 (m, 2H), 3.92 – 3.89 (m, 2H), 3.87-3.78 (m, 2H), 3.74 (d, J = 5.0 Hz, 1H), 3.63 (d, J = 3.2 Hz, 1H), 3.62-3.56 (m, 2H), 3.52-3.43 (m, 5H), 3.42-3.31 (m, 3H), 3.25 (dd, J = 13.6, 7.4 Hz, 2H), 3.02 (d, J = 1.6 Hz, 3H), 2.93 (s, 3H), 2.54 – 2.47 (m, 1H), 2.37 (t, J = 7.3 Hz, 2H), 2.20-1.92 (m, 4H), 1.71-1.60 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 179.57, 160.80, 148.33, 143.56, 138.22, 136.40, 130.75, 130.67, 130.62, 129.24, 128.45, 128.10, 124.31, 123.62, 119.70, 116.12, 111.34, 96.32, 96.26, 85.93, 82.39, 76.02, 74.48, 73.58, 72.18, 71.51, 70.70, 69.14, 68.85, 68.38, 61.38, 58.42, 54.84, 52.03, 51.15, 49.69, 41.60, 37.17, 34.55, 29.62, 29.11, 22.50, 20.74, 18.93; MS (MALDI-TOF) m/z Calcd for $\text{C}_{52}\text{H}_{80}\text{N}_9\text{O}_{13}\text{S}_2$: 1102.53 ($\text{M}+\text{H}$) $^+$, Found: 1102.310, HRMS (ESI-TOF) m/z Calcd for $\text{C}_{52}\text{H}_{80}\text{N}_9\text{O}_{13}\text{S}_2$: 1102.5317 ($\text{M}+\text{H}$) $^+$, Found: 1102.5328; UV (0.008 mM in DI water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 8.40 min, purity 99%.

Compound 11 (DPA 548)

(6 mg, 86%); ^1H NMR (500 MHz, Acetone- d_6) δ 8.26-8.23 (m, 5H), 8.12 (s, 2H), 8.10 – 7.92 (m, 2 H), 5.73 (d, J = 3.74 Hz, 1H), 5.21 (d, J = 4.04 Hz, 1H), 4.76 (s, 1H), 4.26 (d, J = 6.5 Hz, 3H), 3.98-3.52 (m, 7H), 3.40 - 3.12 (m, 8H), 2.72-2.62 (m, 4H), 2.48-2.28 (m, 3H), 1.78-1.72 (m, 1 H), 1.24-1.15 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ 179.99, 161.22, 148.75, 143.98, 138.65, 136.82, 131.17, 131.05, 129.66, 128.88, 128.53, 124.73, 124.04, 120.12, 116.55, 111.77, 96.69, 86.35, 82.80, 76.44, 74.91, 74.00, 72.60, 71.94, 71.13, 69.57, 69.28, 68.80, 61.81, 58.84, 55.26, 52.46, 51.57, 50.11, 42.02, 37.60, 34.98, 30.04, 29.54, 22.93, 21.16, 19.36; MS (MALDI-TOF) m/z Calcd for $\text{C}_{42}\text{H}_{59}\text{N}_7\text{O}_{13}\text{Na}$: 892.96 ($\text{M}+\text{Na}$) $^+$, Found: 893.23; HRMS (ESI-TOF) m/z Calcd for $\text{C}_{42}\text{H}_{60}\text{N}_7\text{O}_{13}$: 870.4249 ($\text{M}+\text{H}$) $^+$, Found: 870.4227; UV (water): $\lambda_{\text{max}1}$ (nm) = 239, $\lambda_{\text{max}2}$ (nm) = 274, $\lambda_{\text{max}3}$ (nm) = 342; HPLC t_R = 6.73 min, purity 96%.

rRNA A-site synthesis

The following 27-base A-site rRNA sequences were used in this study:

<i>E. coli</i> :	5'-GGCGUCACACCUUCGGGUGAAGUCGCC-3'
human cytosolic:	5'-GGCGUCGCUCCUUCGGGAAAAGUCGCC-3'
mitochondrial:	5'-GGCGUCACCCCUUCGGGACAAGUCGCC-3'
C1410U:	5'-GGCGUCACUCCUUCGGGACAAGUCGCC-3'
A1490G:	5'-GGCGUCACCCCUUCGGGCAAGUCGCC-3'

The A-site RNA homologues were synthesized using standard phosphoramidite solid-phase synthesis with a 2'-ACE protecting group (Thermo Scientific). All RNA oligonucleotides were deprotected before use according to the manufacturer's protocol, and the deprotection buffer was removed by evaporation using a SpeedVac (GeneVac). The RNA oligonucleotides were resuspended in diethylpyrocarbonate (DEPC) treated distilled-deionized water to the desired concentration, and the concentrations of oligonucleotides were determined by

reading absorbance at 260nm and 10-mm pathlengths with a Nanodrop 2000c (Thermo Scientific) using an extinction coefficient provided by the manufacturer.

Screening for binding affinity of PYR-NEO conjugates against A-site rRNA models

The methodology for the F-NEO (Figure 3) displacement assay for screening binding affinity of novel compounds has been described previously.²² Briefly, equal volumes (100 μ L) of the F-NEO:A-site rRNA complex (F-NEO:A-site) and PYR-NEO conjugates were added to the wells of a 96-well plate. Final concentration of the F-NEO:A-site was 100 nM in 10 mM HEPES, 50 mM NaCl, and 0.04 mM EDTA (pH 7.0). The control experiments included the F-NEO:A-site without added PYR-NEO conjugate and negative control with test PYR-NEO conjugate without F-NEO:A-site. Before scanning, plates were incubated for 10-20 min at room temperature. Fluorescent emission intensity readings were performed using the TECAN M1000Pro plate reader (λ_{ex} =485 nm, λ_{em} =525 nm). Binding assays were performed in duplicate and the percent binding of PYR-NEO conjugates relative to NEO was calculated as follows:

$$\text{Percent Binding} = 100 \times \frac{(I_{is} - I_c)}{I_{neo} - I_c}$$

Where I_{is} is the intensity of the complex with added PYR-NEO, I_c is the intensity of the control without PYR-NEO, and I_{NEO} is the intensity of the complex with added NEO.

Determination of IC₅₀ values of PYR-NEO conjugates with the A-site RNA

IC₅₀ values were determined by titrations of 100 nM F-NEO:A-site complex with PYR-NEO conjugates. All PYR-NEO conjugates, except compound **13**, were serially diluted two-fold from 20 to 0.009 μ M. For higher IC₅₀ values, titrations were started at a higher concentration range from 60 μ M, 40 μ M, and 20 μ M concentrations and consecutively diluted twice from 20 μ M solution nine times. The IC₅₀ values were calculated using OriginPro 2016 software using the curve fitting *DoseResp* function.

Determination of selectivity factor

Selectivity factor compares the relative affinity of ligands to *E. coli* RNA A-sites compared to human A-site targets. First, the IC₅₀ values were determined, then the selectivity factors were calculated and compared to NEO.²⁵

$$\text{Selectivity Factor} = 5.75 \times \frac{IC_{50}(\text{human})}{IC_{50}(E. coli)}$$

Testing of AME activities against PYR-NEO conjugates

To determine if various AMEs would have the power to inactivate our PYR-NEO conjugates, we used standard assays to visualize the modification of compounds **10-16**. We used NEO as a control. All AME enzymes, AAC(6')-Ie,³⁴ AAC(3)-IV,³⁴ AAC(2')-Ic,³⁵ Eis,³⁵ APH(2'')-Ia,³⁶ and APH(3')-Ia,³⁷ were purified and tested as previously described. All reactions were monitored at 25 °C (with the exception of AAC(6')-Ie and APH(2'')-Ia,

which were monitored at 37 °C) on a SpectraMax M5 microplate reader and performed in duplicate. All rates were normalized to NEO.

Acetylation

The activity of the acetyltransferases was monitored using Ellman's method, coupling the release of the product (CoASH) with DTNB and monitored at 412 nm (ϵ 14,150 cm⁻¹M⁻¹). Briefly, reactions (200 μ L) containing PYR-NEO conjugate (100 μ M) and AcCoA (500 μ M for Eis and 150 μ M for all other acetyltransferases) were incubated with the enzymes (0.125 μ M for AAC(3)-IV and AAC(2')-Ic; 0.5 μ M for all remaining acetyltransferases) in the presence of DTNB (2 mM) and the appropriate buffer (50 mM MES pH 6.6 for AAC(6')-Ie and AAC(3)-IV, 50 mM Tris-HCl pH 7.5 for AAC(6')-Ib, 100 mM sodium phosphate pH 7.4 for AAC(2')-Ic, and 50 mM Tris-HCl pH 8.0 for Eis). Using kinetic measurements, reading every 30 s for 30 min, initial rates of the reactions were calculated using the first 2 min of the reaction.

Procedure for translation inhibition activity determination

The prokaryotic translation inhibition activities of representative compounds were determined using an assay based on *Escherichia coli* cell extract (*E. coli* S30 Extract System for Circular DNA, Promega, LI020) as recommended by the manufacturer.^{38–39} Briefly, stock solutions of each compound were made in DMSO by serial dilution with the final dose range of 3.73 μ M to 5.12 nM. Various concentrations of each compound were allowed to incubate in a solution of cellular extract and all amino acids for 20 min at room temperature. After brief centrifugation, 0.40 μ L of luciferase control template (Promega, L492A-C) was added to each tube. After gentle mixing and spin down, tubes were incubated at 37 °C for 60 min. The reaction was terminated by inactivating on ice for 5 min. Upon thawing to room temperature, 5 μ L per tube was delivered to a LUMITRAC™ 200 96-well plate. Luminescence was read immediately after the addition of the luciferin solution using a Molecular Devices SpectraMax M2. IC₅₀ values were determined by nonlinear fit using GraphPad Prism 6. All compounds were analyzed in triplicate and standardized against an internal vehicle control.

Bacterial strains and culture conditions

The characteristics of the bacterial strains used in this study are given in Table 4. *Enterococcus faecalis*, *Enterococcus faecium*, and *Streptococcus pyogenes* were cultured in Brain-Heart Infusion (BHI) broth. *Mycobacterium smegmatis* was cultured in Tryptic Soy (TS) broth supplemented with 0.05% Tween 80 (to reduce cell aggregation). TS broth was used for culturing of all other strains. Purity of all bacterial strains was verified on TS agar plates.

Initial antibacterial screening and minimal inhibitory concentration (MIC) determination

Initial screening of PYR-NEO conjugates against exponential phase microbial cultures was performed at a 6.25 μ M concentration of each compound performed in duplicate using 96-well polystyrene microplates along with the reference compound NEO. The same volume (10 μ L) of sterile water with 1% DMSO used for antibiotic addition was added to the broth

(background control) and microbial culture (growth control). Plates were incubated in a humidified incubator at 37 °C for 15-20 h. The percent growth inhibition was calculated using the formula:

$$\%Growth\ Inhibition = 100 - 100 \times \frac{A_{compound} - A_{background}}{A_{control} - A_{background}}$$

MIC values for select PYR-NEO conjugates were determined for exponential phase bacterial cultures by the microdilution method in triplicate according to the Clinical and Laboratory Standards Institute (CLSI) protocol.⁴⁰ Stock test compounds were prepared at 10× the final concentration in 10% DMSO. For each dilution, a 10 µL aliquot of stock test compound was combined with 90 µL diluted bacterial suspension (~3×10⁵ cells per mL cation-adjusted Mueller-Hinton broth). Final test compound concentrations from serial 2-fold dilutions ranged from 50 µM to 0.78 µM. Plates were incubated in a humidified incubator at 37 °C for 15-20 h. After incubation with test compound growth was measured by absorbance at 595 nm using a TecanM100Pro plate reader. The percent growth inhibition at each concentration was averaged from triplicate assays calculated from the formula above.

Docking experiments

The docking experiments were performed using NMR based prokaryotic (PDB ID: 1PBR)³¹ and eukaryotic (PDB ID: 1FYO)⁴¹ A-site receptor structures. In the case of prokaryotic A-site structure, the bound ligand was manually removed prior to receptor preparation using AutoDock Tools (version 1.5.6). Three lead molecules **11-13** (based on IC₅₀ and selectivity factor values) were chosen for docking studies. All docking experiment were run as 'blind experiment' (blind experiment refers to the use of a grid box, which encompasses the entire receptor structure to allow determination of any mode of possible ligand-receptor complexation) using AutoDock Vina 1.1.2.⁴² AutoDock Vina was chosen considering its high accuracy with ligands possessing more than 20 rotatable bonds (the three ligands used had >20 rotatable bonds in each case). AutoDock Vina docking was performed using an exhaustiveness value of 8. All rotatable bonds within the ligand were allowed to rotate freely. All ligand structures were created using ChemDraw Ultra10.0 and then energy minimized in Chem3D. They were then further minimized energetically by the Vega ZZ program⁴³ utilizing a conjugate gradient method with AutoDock force field. Further conversions of receptor and ligand PDB structures for docking using AutoDock Vina was performed with AutoDock Tools version 1.5.6. After the docking experiments, the structures with most favorable energies were used for final rendering with PyMol (www.pymol.org).

Drug toxicity in *C. elegans*

C. elegans was maintained according to standard practices.⁴⁴⁻⁴⁹ Toxicity assays were performed in three separate 96 well microtiter plates with 50 µL final volume of worm suspension. L1-stage worms were transferred to wells (500 worms/well) containing 5 µL of a 10X solution of each control or test compound at each 1:2 serial dilution. The concentration range of the control drug neomycin and test compounds was 50 – 1.56 µM and were administered in triplicate in each of the three microtiter plates for a total of 9 replications.

Viability was determined using the MTT assay after 24 h drug exposure time at 20°C. The MTT assay was performed as follows; L1-stage worms were diluted in M9 buffer to approximately 10⁵ worms/mL. and 45 µL aliquots containing 500 worms were added to the wells of a 96 well microtiter flat bottom plate with and without the control drug neomycin or test compound. After the 24 h treatment period, 50 µL of a 10 mg/mL solution of MTT in phosphate buffered saline (pH 7) was added for a final assay concentration of 5 mg/mL. Plates were incubated for 3 h at 20°C in a temperature-controlled chamber. The contents of each well were transferred to a microfuge tube and the worms were pelleted by centrifugation at 2500 rpm and the supernatant aspirated. DMSO (50 µL) was added to each tube to lyse the worm pellet and solubilize the formazan crystals, incubated for 1 h at room temperature and then transferred back to the 96 well microtiter plate. Formazan production in each well was quantified by absorbance at 575 nm using a TECAN PRO 1000 plate reader. The worms exposed to nine replicates for each compound at each concentration were assayed for formazan production. The differences between treatments and the untreated control was determined using the student *t* test. Significant difference was determined at *P*<0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

We thank the National Institute of Health for financial support (grants GM097917, AI111414 to D.P.A.) and (grants AI090048 to S.G.-T.).

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Syntheses of aromatic-aminosugar conjugates in high yields

Linker length dependence of conjugate binding to ribosomal RNA

Much improved Gram positive inhibition in aminoglycoside resistant MRSA strains

Ability to evade aminoglycoside modifying enzymes

Scheme 1.

Synthesis of PYR-NEO conjugates **10-16**. Reaction conditions and yields: (a) **1-3**, *N*-hydroxysuccinimide, EDC, 1,4-dioxane, 24 h, room temperature, 82-90%. (b) **6**, 1,6-hexanediamine, 1,4-dioxane, 12 h, room temperature, 54%. (c) TCDP, DMAP, Py, 6 h, room temperature, 79%. (d) DMAP, Py, 10 h, room temperature, 59-73%. (e) TFA, CH_2Cl_2 , 30 min, room temperature, 81-88%.

Figure 1.

Structures of PYR-NEO conjugates **10-16** used in this study. Compound purity was verified by RP-HPLC and HPLC purity profiles are included in the Supporting Information.

Figure 2.
A-site secondary structures used in PYR-NEO study highlighting differences in the bulge regions.

Figure 3.
Structure of F-NEO, a molecular reporter of compound binding.

Figure 4.
AME modification of PYR-NEO conjugates relative to NEO.

Figure 5.

Models of compound **11** in lowest energy conformations when bound to (a) bacterial and (b) human A-site RNA structures. The RNA bases are colored as: adenine (red), guanine (green), cytosine (purple) and uracil (orange) The pyrene moiety in compound **11** is colored black. The boxed region shows the aminoglycoside binding pocket in both the structures.

Figure 6.

Pyrene-neomycin conjugates DPA 543 and 544 were evaluated for toxicity at a concentration range of 50 – 1.56 μM as compared to the neomycin control. Compounds were tested on L1-stage *C. elegans* and viability was assessed using the MTT assay where formazan production was measured by absorbance at 575 nm after 24 h incubation with each compound.¹ NT: no treatment, NEO: neomycin. The results are presented as an average of 9 replicates. Error bars represent the standard deviation from the mean.

Table 1.

Percent displacement of F-NEO by PYR-NEO conjugates upon addition to A-Site rRNA:F-NEO complex, relative to NEO.

Compound (linker length)	<i>E. coli</i>	human	mitochondrial	C1410U	A1490G
NEO	100 ± 8	100 ± 6	100 ± 3	100 ± 3	100 ± 6
10 (4)	33 ± 1	38 ± 14	47 ± 1	73 ± 4	22 ± 12
11 (5)	33 ± 5	39 ± 1	47 ± 1	65 ± 54	22 ± 7
12 (6)	99 ± 6	99 ± 14	89 ± 2	103 ± 1	88 ± 5
13 (7)	101 ± 2	114 ± 7	102 ± 7	103 ± 2	102 ± 10
14 (8)	88 ± 4	91 ± 5	88 ± 5	86 ± 10	65 ± 3
15 (9)	78 ± 10	66 ± 18	74 ± 7	92 ± 1	58 ± 20
16 (18)	64 ± 2	75 ± 2	70 ± 1	91 ± 1	58 ± 6

Table 2.IC₅₀ values of PYR-NEO conjugates to rRNA A-sites.

Compound (linker length)	<i>E. coli</i>	human	mitochondrial	C1410U
10 (4)	303 ± 2	375 ± 9	339 ± 6	274 ± 20
11 (5)	310 ± 1	447 ± 18	373 ± 2	332 ± 2
12 (6)	170 ± 2	200 ± 36	465 ± 35	149 ± 11
13 (7)	95 ± 2	97 ± 3	90 ± 2	70 ± 5
14 (8)	191 ± 1	204 ± 2	211 ± 18	147 ± 15
15 (9)	148 ± 4	175 ± 1	168 ± 1	126**
16 (18)	254 ± 1	252 ± 4	235 ± 4	180 ± 1

Table 3.Selectivity factors of PYR-NEO series to *E. coli* and the human cytosolic A-site.

Compound	IC ₅₀ (<i>E. coli</i>)	IC ₅₀ (human)	Selectivity factor
NEO	87	76	5.02
10 (4)	303	375	7.12
11 (5)	310	447	8.29
12 (6)	170	200	6.76
13 (7)	95	97	5.87
14 (8)	191	204	6.14
15 (9)	148	175	6.80
16 (18)	254	252	5.70

Table 4.

Characteristics of bacterial strains used in this study.

Strain	Relevant characteristics
Gram-positive	
<i>Staphylococcus aureus</i> 25923	NEO(S)
<i>Staphylococcus aureus</i> NRS77	NEO(S)
<i>Staphylococcus aureus</i> 6538	NEO(S)
<i>Staphylococcus aureus</i> NorA	NEO(S), <i>norA</i> , efflux mutant
<i>Staphylococcus epidermidis</i> 12384	NEO(S)
MRSA 33591	NEO(R), multidrug-resistant
MRSA A960649	NEO(R), multidrug-resistant
MRSA SU-5	NEO(R), multidrug-resistant
MRSA M0602	NEO(R), multidrug-resistant
<i>Streptococcus pyogenes</i> C203	NEO(R)
<i>Enterococcus faecalis</i> 29212	NEO(IM)
<i>Enterococcus faecium</i> BM4105-RF	NEO(IM)
<i>Bacillus anthracis</i> BA852	NEO(S)
<i>Listeria monocytogenes</i> 19115	NEO(S)
<i>Mycobacterium smegmatis</i>	NEO(S)
Gram-negative	
<i>Escherichia coli</i> 25922	NEO(S)
<i>Escherichia coli</i> TolC	NEO(S), <i>tolC</i> , efflux mutant
<i>Escherichia coli</i> H4H	NEO(R), multidrug-resistant
<i>Enterobacter cloacae</i> 13047	NEO(S)
<i>Acinetobacter baumannii</i> 19606	NEO(S)
<i>Pseudomonas aeruginosa</i> 27853	NEO(R)
<i>Salmonella enterica</i> NR-12171	NEO(S)
<i>Shigella sonnei</i> NR-519	NEO(S)
<i>Proteus mirabilis</i> HM-752	NEO(IM)
<i>Klebsiella oxytoca</i> HM-625	NEO(S)
<i>Klebsiella pneumoniae</i> NR-151410	NEO(IM)
<i>Yersinia pestis</i> NR-4689, A12	NEO(S)

Abbreviations: NEO(R), NEO(IM), and NEO(S) refer to NEO-resistant, NEO-intermediate sensitivity, and NEO-sensitive, respectively.

Table 5.

Minimal inhibitory concentration (MIC) values for NEO and select PYR-NEO conjugates.

Strain	NEO	12	13	15	16
<i>S. aureus</i> 25923	1.56	12.5	12.5	12.5	6.25
<i>S. aureus</i> NorA	1.56	12.5	6.25-12.5	6.25-12.5	6.25
<i>S. epidermidis</i> 12384	1.56	12.5	6.25	6.25	3.13
MRSA 33591	400	12.5	12.5	12.5	25
MRSA A960649	100	12.5	12.5	12.5	25
MRSA SU-5	200-400	25	25	25	50
MRSA M0602	>400	25	25	25	25-50
<i>S. pyogenes</i> C202	NA	25	NA	50	NA
<i>B. anthracis</i> BA852	1.56	12.5	6.25	6.25	3.13
<i>M. smegmatis</i>	0.78	12.5	6.25	6.25	6.25
<i>E. coli</i> 25923	3.13	>25	>25	>25	>25
<i>E. coli</i> TolC	1.56	12.5	6.25	6.25	1.56-3.13
<i>E. coli</i> H4H	400	>25	>25	>25 [*]	>25 [*]

MIC values were determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI). MIC values (μM) were identified from triplicate assays.

^{*} A 30% reduction in growth was observed for compounds **15** and **16** with *E. coli* H4H.