ChemPubSoc Europe

DOI: 10.1002/cbic.201500256

CHEMBIOCHEM Communications

Tobramycin Variants with Enhanced Ribosome-Targeting Activity

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With the increased evolution of aminoglycoside (AG)-resistant bacterial strains, the need to develop AGs with 1) enhanced antimicrobial activity, 2) the ability to evade resistance mechanisms, and 3) the capability of targeting the ribosome with higher efficiency is more and more pressing. The chemical derivatization of the naturally occurring tobramycin (TOB) by attachment of 37 different thioether groups at the 6″-position led to the identification of generally poorer substrates of TOB-targeting AG-modifying enzymes (AMEs). Thirteen of these displayed better antibacterial activity than the parent TOB while retaining ribosome-targeting specificity. Analysis of these compounds in vitro shed light on the mechanism by which they act and revealed three with clearly enhanced ribosome-targeting activity.

Aminoglycosides (AGs) represent one of the major groups of antibiotics that target the bacterial ribosome. They interfere with translation,[1] promote errors during decoding,[2] and inhibit translocation[3] and ribosome recycling.[4] AGs have been shown to target the decoding site of the 30S subunit, interacting at the internal loop of helix h44 in which A1408 lies across from A1492 and A1493.[5] The most structurally conserved portion of the AGs (rings I and II) forms most of the contacts to h44. Ring I intercalates into h44, stacking on G1491 and forming hydrogen bonds with A1408. This occludes A1492 and A1493 from within h44 and hence stabilizes a "flipped out" conformation of these nucleotides. An analogous rearrangement occurs upon codon recognition during decoding: A1492 and A1493 flip out of h44 and dock into the minor groove of the codon–anticodon helix.[6] The ability of AGs to stabilize this conformation of h44 thus appears to disturb translational fidelity during protein synthesis. AG binding pays the energetic cost for the rearrangement and thereby stabilizes tRNA in the A site. This leads to miscoding, because near-cognate aa-tRNA is also stabilized.[2] This also inhibits translocation, because A-tRNA cannot readily move from the A to the P site.[3,7]

The clinical usefulness of AGs has been seriously compromised by the growing prevalence of various resistance mechanisms among pathogenic bacteria. These mechanisms include the decrease in AG uptake into the bacteria, the alteration of the bacterial ribosome, and the acquisition of AG-modifying enzymes (AMEs), which represent the major cause of resistance to AGs.[8] With more than 100 AMEs identified, these enzymes pose a serious health threat as they chemically alter the structures of AGs by N-acetylation (AACs), O-phosphorylation (APHs), or O-nucleotidylation (ANTs). To overcome this issue, AGs that could evade the action of AMEs while still targeting bacterial ribosomal RNA have been investigated. This has led to the development of structurally constrained AGs that would mimic the ribosome-bound AG conformation,[9] guanidinylated AGs,[10] and AG dimers,[11] which have been shown to also bind viral and human RNAs.[12]

We previously synthesized a number of 6″-thioether tobramycin (TOB) variants and assayed their antimicrobial activities.[13] Many of these compounds exhibited bacteriolytic activity, raising the possibility that the mode of action in these cases involves membrane disruption rather than translation inhibition. Herein, we present the synthesis of 18 additional TOB variants (Scheme 1), establish their antibacterial activity profile, and investigate the mechanism by which the 18 new and the 19 previously reported 6″-thioether TOB variants inhibit bacterial growth, by using AG-resistant ribosomes with mutations in the primary helix h44 site.

TOB was modified at the 6″-position with various thioether groups (Scheme 1). Whereas compounds **3a**–**j**, **3l**, **3m**, **3p**–**t**, **3jj**, and **3kk** have been described previously,[13–14] compounds **3k**, **3n**, **3o**, and **3u**–**ii** are new. Our collection encompasses a diverse set of 6″-substituents, including linear, branched, and cyclic alkyl groups, and substituted aromatic rings.

Compounds **3a**–**f**, **3k**–**p**, and **3u**–**jj** were screened for their antibacterial activity against 19 diverse bacterial strains, and their minimum inhibitory concentrations (MICs) were determined (Table S1 in the Supporting Information). Among the variants with aliphatic substituents, compound **3f** (with a C_{14} chain) was the most potent against several of the TOB-resistant bacterial strains, including the newly tested *Enterococcus faecium* (**C**) and *Streptococcus pyogenes* (**L**). Compounds bearing aromatic substituents generally exhibited promising antibacterial activity (MIC $\leq$ 9.4 μg mL^{-1}) against *Bacillus anthracis* (**A**), *Bacillus subtilis* (**B**), *Listeria monocytogenes* (**E**), *Mycobacterium smegmatis* (**I**), *Staphylococcus aureus* NorA (**J**), *Staphylococcus epider-*

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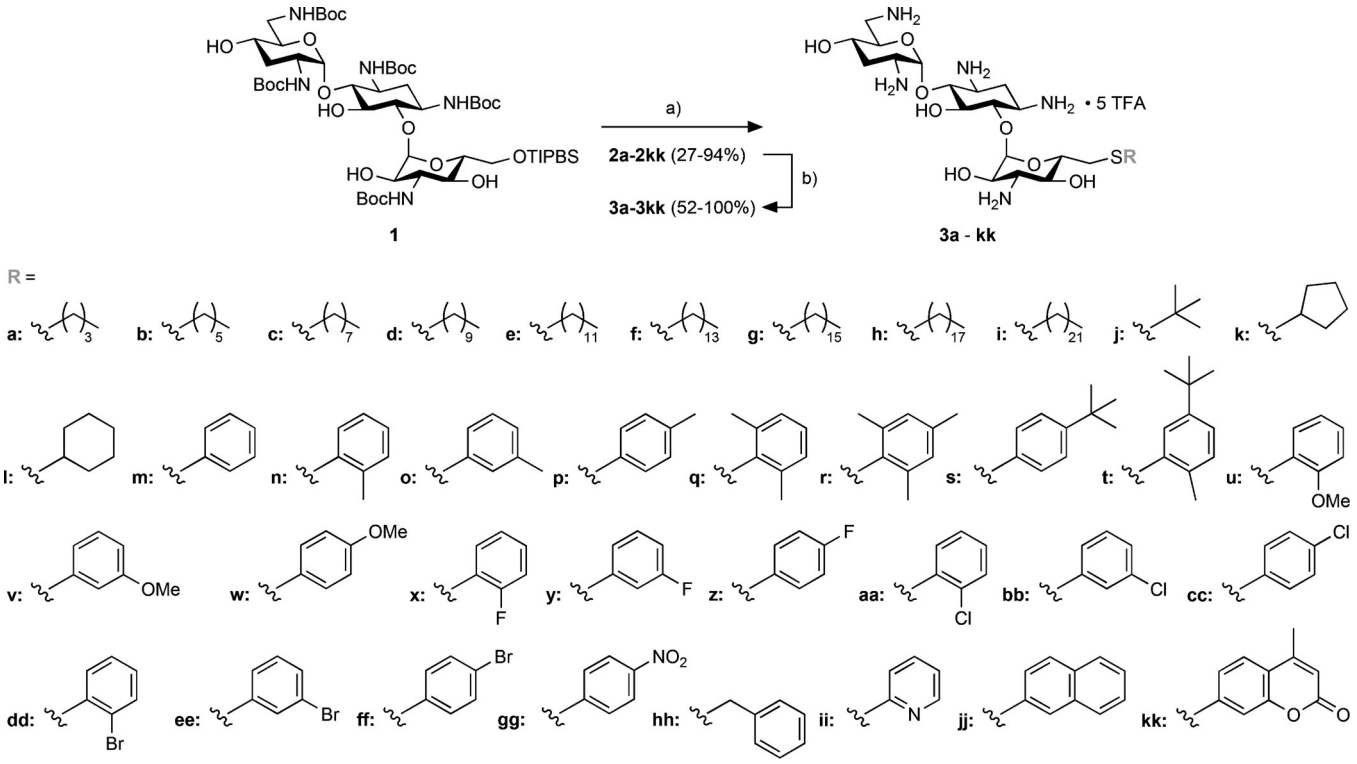
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201500256.



Scheme 1. Synthesis of 6″-thioether TOB variants. a) RSH, Cs_2CO_3, DMF; b) TFA.

midis (**K**), *Escherichia coli* (**N**), *Haemophilus influenzae* (**H**), and *Shigella flexneri* (**S**). Several of these, including compounds **3m**–**n**, **3p**, **3x**–**z**, **3aa**, **3cc**–**dd**, **3ff**, **3gg**, and **3ii** were even capable of inhibiting the growth of various bacterial strains at an MIC $\leq$ 2.4 μg mL^{-1}. Furthermore, additional substitution at the *ortho*- or *para*-positions on the aryl ring appeared to improve antibacterial activity. It is worth mentioning that compounds **3m** (MIC 2.4 μg mL^{-1}), **3p** (MIC 1.2 μg mL^{-1}), and **3jj** (MIC 9.4 μg mL^{-1}) displayed a 16- to 128-fold decrease in MIC value compare to TOB (MIC > 150 μg mL^{-1}) against *E. coli* overexpressing TolC (**O**), whereas compound **3z** (MIC 18.8 μg mL^{-1}) was eight times more active than TOB (MIC 150 μg mL^{-1}) against *Mycobacterium intracellulare* (**G**).

As these compounds demonstrated comparable or better potency against *E. coli* strains, we further investigated their mechanisms of action. We compared their abilities to inhibit growth of *E. coli* Δ7 prrn containing wild-type (WT), A1408G, or G1491U ribosomes. Strain Δ7 prrn lacks all seven chromosomal rRNA operons and instead contains a single plasmid-borne rRNA operon.[15] Hence, each of these strains contains a homogeneous population of WT or mutant ribosomes. Mutations A1408G and G1491U target the primary AG binding site of helix h44 of the 30S subunit, and each mutation confers resistance to a number of AGs.[16]

TOB inhibited *E. coli* Δ7 prrn WT (MIC 18.8 μg mL^{-1}) and failed to inhibit either Δ7 prrn A1408G or G1491U (MIC > 150 μg mL^{-1}; Table S2), indicating that TOB inhibits growth by binding its h44 site, consistent with previous results.[16a,c] Many of the TOB variants showed a similar activity profile against these strains, inhibiting growth in an A1408- and G1491-dependent manner. Several of these (**3m**–**n**, **3p**, **3x**–**z**, **3aa**–**dd**, **3ff**–**hh**), all carrying an aryl ring substituent, were more potent than TOB and retained target specificity.

Two compounds (**3f** and **3g**) were found to have indistinguishably strong antibacterial activity against Δ7 prrn WT, A1408G, and G1491U, showing that these compounds act through a distinct mechanism, independent of h44. These compounds, substituted with linear alkyl chains (C_{14} and C_{16}, respectively) were previously found to have cellulolytic activity,[13] which might be their primary mechanism of action. Compounds **3d** and **3e**, with slightly shorter alkyl chains (C_{10} and C_{12}, respectively), have lower activity with little to no h44-dependence.

Finally, a number of compounds were found to be virtually inactive against all the tested strains. These include compounds **3b**, **3c**, **3h**, and **3i** (substituted with C_6, C_8, C_{18}, or C_{22} linear alkyl chains, respectively), compound **3s** (modified with a 4-*tert*-butyl thiophenyl group), and compound **3kk** (with a 4-methylcoumarin-7-yl group).

Some AGs are potent inhibitors of EF-G-dependent translocation.[3,7,17] This is believed to be due to the stabilization of A-site tRNA, which occurs when these antibiotics bind their primary site in h44. We suspected that TOB would also inhibit translocation, allowing effects of the 6″-substituents to be compared in vitro. To test this, we purified WT and A1408G ribosomes from the corresponding Δ7 prrn strains and used toeprinting to measure the extent of translocation in the presence of various concentrations of TOB. The resulting data were fitted to the Hill equation. TOB strongly inhibited translocation of WT ribosomes (IC_{50} 16 μM; Figure 1, Table 1). Interestingly,

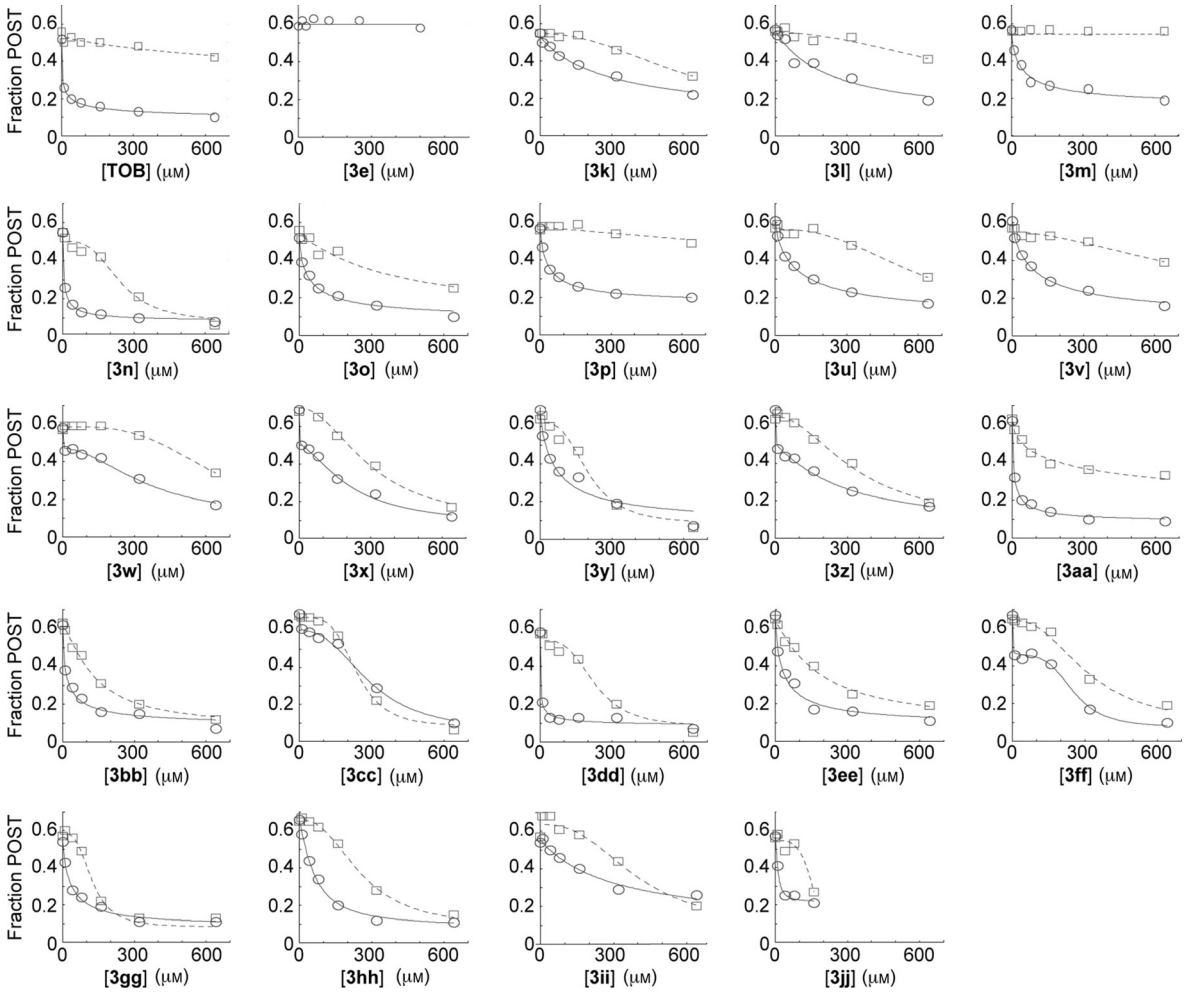


Figure 1. Effects of TOB variants on ribosomal translocation. The extent of EF-G-dependent translocation was measured in control (WT; ○) and mutant (A1408G; □) *E. coli* ribosomes with various concentrations of TOB variants.

Table 1. Translocation inhibition activities of TOB variants in wild-type (WT) or mutant A1408G *E. coli* ribosomes.

AG	IC_{50} [μM] Control (WT)	A1408G	AG	IC_{50} [μM] Control (WT)	A1408G
TOB	16	700	**3d**	>1000	n.d.
3e	>1000	n.d.	**3h**	>1000	n.d.
3k	330	660	**3l**	230	>1000
3m	44	>1000	**3n**	5	250
3o	47	430	**3p**	36	>1000
3u	100	620	**3v**	100	990
3w	<1; 360[a]	650	**3x**	<1; 210[a]	310
3y	72	210	**3z**	<1; 280[a]	350
3aa	7.7	370	**3bb**	19	140
3cc	<5; 290[a]	240	**3dd**	1.2	230
3ee	33	180	**3ff**	<1; 250[a]	320
3gg	39	130	**3hh**	66	270
3ii	350	410	**3jj**	11	100

[a] Curves in these cases exhibited complex concentration dependence. Data were fitted to the sum of two Hill functions, and the reported values correspond to the two deduced inflection points (IC_{50} values). n.d. = not determined.

the Hill coefficient derived from the curve fitting was consistently less than 1 (0.5–0.7), raising the possibility that TOB binds its primary (h44) and secondary (H69)[5a] sites in a negative cooperative manner. With A1408G ribosomes, TOB had substantially reduced potency (IC_{50} 700 μM), and the data could be well fit with a Hill coefficient of either 1 or >1, depending on the particular experiment. These data show that inhibition of translocation by TOB normally depends on the primary h44 site, and mutation of that site qualitatively changes the concentration dependence of inhibition.

Next, the effects of several TOB variants on translocation were analyzed (Figure 1, Table 1). Many behaved similarly to the parent compound, although differences correlating with the structure of the substituent were observed. Four compounds inhibited translocation more strongly than TOB in both WT and A1408G ribosomes. Three of these (**3n**, **3aa**, **3dd**) have *ortho*-substituted thioaryl groups, whereas the fourth carries a naphthyl moiety (**3jj**). For the former compounds, IC_{50} values similarly decreased for WT and mutant ribosomes. Compound **3jj**, on the other hand, exhibited some loss of specifici-

ty, as the ratio of IC_{50} values (WT vs. A1408G) dropped from 44 to 9. Compounds **3u** and **3x** (with *o*-methoxythiophenyl or *o*-fluorothiophenyl groups) were notably poorer inhibitors of translocation.

Compounds with *meta*-substituted thioaryl groups (**3o**, **3v**, **3y**, **3bb**, **3ee**) were generally less potent translocation inhibitors than their *ortho*-substituted counterparts and had reduced specificity for WT ribosomes (Figure 1, Table 1). As with the *ortho*-substituted set, the methoxythiophenyl- and fluorothiophenyl-modified compounds (**3v** and **y**) were the weakest inhibitors of the group.

Many of the compounds with *para*-substituted aryl rings (**3w**, **3z**, **3cc**, **3ff**) gave complex inhibition curves that failed to fit the standard Hill equation (Figure 1). In these cases, the lowest concentration of drug tested (10 μM) resulted in a substantial degree of inhibition, and further inhibition occurred only gradually over the higher range of drug concentrations. Although the basis of this complexity remains unclear, we speculate that it might arise from two distinct populations of ribosomal complexes, one being considerably more sensitive to AG inhibition than the other. Accordingly, the data were fitted to the sum of two Hill functions, yielding inflection points (i.e., IC_{50} values) for the two putative distinct populations (Table 1). In the context of the cell, potent inhibition of even a small subset of translating ribosomes would have deleterious consequences, consistent with the enhanced biological activity of these variants (Table S2). Compound **3x**, which carries an *ortho*-fluorothiophenyl substituent, showed a similarly complex inhibition curve (Figure 1). Finally, compounds **3d**, **3e**, and **3h**, with linear alkyl chains, failed to inhibit translocation in vitro (Figure 1, Table 1).

Compounds **3n**, **3aa**, and **3dd**, with *ortho*-substituted aryl groups, thus appear to be more potent inhibitors of both bacterial growth and ribosomal translocation than TOB in *E. coli* strains. In these cases, inhibition of ribosomes in vivo and in vitro remains strictly dependent on A1408, showing that higher potency comes without loss of target specificity. These TOB variants thus bind the primary h44 site with increased affinity. The way in which these 6′′-aryl substituents promote TOB binding remains to be determined. Co-crystal structures of ribosomes bound to related AGs provide some clues to the basis of enhanced binding.[5a] Rings I and II of these TOB variants make similar sets of contacts to nucleotides 1407–1409 and 1491–1495 of helix h44. Ring III of gentamicin contacts nucleotides 1405–1407, forming hydrogen bonds with G1405 and C1407. Presumably, the analogous ring III of TOB occupies a similar position. The 6′′-aryl groups of the TOB variants might form a favorable stacking interaction with a nearby rRNA nucleotide, such as C1404, and thereby stabilize binding.

For the linear alkyl substituted compounds, potency and mechanism of action changed as a function of chain length. Compound **3a**, with the shortest chain (C_4) of the series, had biological activity indistinguishable from TOB, inhibiting cell growth in an A1408- and G1491-dependent manner. When the length of the chain was increased to C_6 (**3b**), antibacterial activity was completely lost (Table S2), presumably due to a steric clash with the ribosome, shedding light on the spatial constraints of the h44 site. Further lengthening of the alkyl chain to C_{10} through C_{16} (**3d**–**g**) restored antibacterial activity, although this activity was h44-independent. Indeed, these compounds do not appear to target the ribosome, as **3f** failed to inhibit in vitro translation,[13] and **3e** failed to inhibit translocation (Figure 1). Rather, these compounds target the cell membrane and probably act through bacteriolysis.[13] Compounds with even longer chains (**3h**, C_{18}; **3i**, C_{22}) lost potency, likely due to diminished bacteriolytic activity.

As quite a number of our TOB variants demonstrated good antibacterial activity, we evaluated their susceptibility to AMEs, which greatly contribute to bacterial resistance to AGs. We measured the relative activities of four AMEs with our TOB variants and compared them to that of TOB itself (Figure 2).

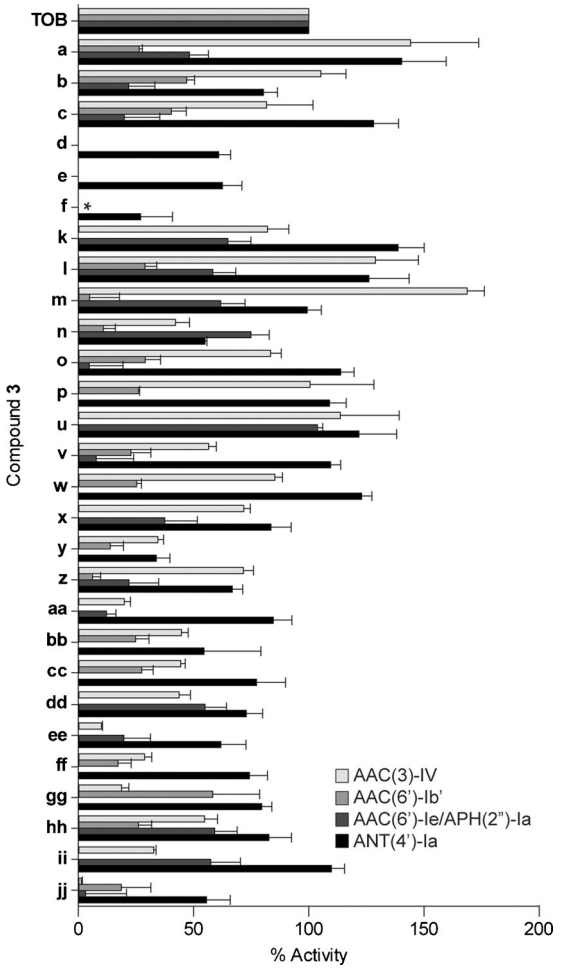


Figure 2. Bar graph showing the relative initial rates of reactions of the listed AMEs with variants **3a**–**jj**. Rates are normalized to TOB. *indicates that **3f** with AAC(6′)-Ib′ had activity >200% and is not shown here.

Although an increase in catalytic activity of AAC(3)-IV[1a] and ANT(4′)-Ia[18] was noticeable in the majority of TOB variants with aliphatic substituents (**3a**–**c**, **3f**, **3k**, **3l**), in general, TOB variants bearing an aromatic ring appeared to be poorer substrates of these AMEs. This implies that TOB variants with aliphatic substituents were more susceptible to modifications by

AAC(3)-IV and ANT(4′)-Ia than TOB variants bearing an aromatic ring. In light of the information gathered from the crystal structures of various AMEs with our parent drug TOB (e.g., AAC(2′)-Ic (PDB ID: 1M4D),[19] APH(2″)-IVa (PDB ID: 3SG8),[20] Eis (PDB ID: 4JD6),[21] along with that of AMEs with other AGs (e.g., AAC(2′)-Ic (PDB ID: 1M4I),[19] AAC(6′)-Ib (PDB ID: 1V0C),[22] APH(2″)-IVa (PDB ID: 3SG9),[20] APH(2″)-Id/APH(2″)-IVa (PDB ID: 4DFB),[23] and APH(3′)-IIIa (PDB ID: 1L8T)[24] with kanamycin A; AAC(2′)-Ic (PDB ID: 1M4G),[19] AAC(6′)-Iy (PDB ID: 1S3Z),[25] and AAC(6′)-Ib (PDB ID: 2BUE)[22] with ribostamycin; AAC(6′)-Ib (PDB ID: 2VQY),[22] and Eis (PDB ID: 4QB9)[26] with paromomycin; APH(3′)-IIIa (PDB ID: 2B0Q)[24] with neomycin B), it appears that the AG binding pocket of AACs is very hydrophilic, lined with several water molecules and amino acid residues with acidic side chains. Replacement of the hydroxy group at position 6″ with a hydrophobic moiety might thus weaken the interactions of the resulting AGs in the binding pocket, reducing their susceptibility to AMEs. Taking all of our combined data into consideration (Figures 1 and 2, Tables 1 and S1), compounds **3 n**, **3 aa**, and **3 dd**, which all contain an *ortho*-substituted thioaryl group, exhibited the most potent inhibitory activity against bacterial growth and ribosomal translation and were also among the poorest substrates for all the tested AMEs. These variants could therefore evade the action of TOB-targeting AMEs AAC(6′)-Ie/APH(2″)-Ia,[1a] AAC(6′)-Ib′,[27] and ANT(4′)-Ia better than TOB. It is important to note that the most common AAC(6′) enzymes were the least active against all variants tested, a highly encouraging result.

In conclusion, 18 novel 6″-thioether TOB variants were synthesized and, together with 19 previously reported TOB derivatives, their antibacterial activities were evaluated. Compound **3 f** (with a C_{14} chain) exhibited the most potent antibacterial activity; meanwhile, compounds bearing aromatic substituents were more active against *E. coli* strains. Importantly, we identified several TOB variants with enhanced ribosome-targeting activity. Compounds **3 n**, **3 aa**, and **3 dd** inhibited bacterial growth and ribosomal translation better than TOB in *E. coli*. These compounds also served as poor substrates for many AMEs, including those known to target parent TOB, suggesting that AMEs will have little-to-no effect on these AG variants. As the addition of aromatic moieties to the 6″-position of TOB appears to be a promising avenue for enhancing ribosome-targeting activity, studies aimed at adding aromatic substituents to AGs at various positions are currently underway in our laboratories.

Acknowledgements

This work was supported by startup funds from the University of Kentucky (S.G.-T.), and by NIH grants AI090048 (S.G.-T.) and GM072528 (K.F.). We thank Aishwarya Devaraj for help with toeprinting experiments.

Keywords: aminoglycoside · antibacterial agents · bacterial resistance · drug-modifying enzymes · translocation

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Manuscript received: May 19, 2015
Accepted article published: May 28, 2015
Final article published: June 17, 2015