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Strategies to overcome the action of aminoglycoside-modifying enzymes for treating resistant bacterial infections

Kristin J Labby and **Sylvie Garneau-Tsodikova***
Associate Professor of Pharmaceutical Sciences, University of Kentucky, College of Pharmacy, Bio Pharm Complex, Room 423, Lexington, KY 40536-0596, USA

Abstract

Shortly after the discovery of the first antibiotics, bacterial resistance began to emerge. Many mechanisms give rise to resistance; the most prevalent mechanism of resistance to the aminoglycoside (AG) family of antibiotics is the action of aminoglycoside-modifying enzymes (AMEs). Since the identification of these modifying enzymes, many efforts have been put forth to prevent their damaging alterations of AGs. These diverse strategies are discussed within this review, including: creating new AGs that are unaffected by AMEs; developing inhibitors of AMEs to be co-delivered with AGs; or regulating AME expression. Modern high-throughput methods as well as drug combinations and repurposing are highlighted as recent drug-discovery efforts towards fighting the increasing antibiotic resistance crisis.

The discovery of antibiotics, compounds that kill or stunt the growth of bacteria, has had a profound impact on human health. Soon after the 1928 discovery of the first antibiotic, penicillin, the first aminoglycoside (AG) antibiotic, streptomycin (STR), was isolated from *Streptomyces griseus* in 1943 and used as the first effective treatment for tuberculosis (TB) [1]. AGs are still commonly used today for broad-spectrum treatment of bacterial infections [2]. The term AG encompasses the family of antibacterial compounds whose structure consists of two or more modified amino-sugars (Figure 1A). AGs act by binding to the A-site of the 16S rRNA subunit of the bacterial ribosome, hindering proper matching of aminoacyl-tRNAs to the anticodon. This leads to the synthesis of aberrant proteins, eventually resulting in bacterial cell death [3]. *Streptomyces* and *Micromonospora* are the bacterial genera that produce AG natural products [4]. These organisms avoid inhibiting their own ribosomes by methylating their 16S RNA, preventing key AG–rRNA interactions [5]. Unfortunately, as with most therapeutics, AGs do have toxic side effects. For example, nonspecific binding of AGs to the eukaryotic ribosome A-site, which only differs from that of prokaryotes by a single base pair (the prokaryotic A1408 corresponds to G1408 in eukaryotes), is one of the causes that lead to toxic side effects including nephrotoxicity and ototoxicity [6,7]. The only AG currently known to not display ototoxicity is apramycin (APR) [8].

*Author for correspondence: Tel.: +1 859 218 1686, Fax: +1 859 257 7585, sylviegtsodikova@uky.edu.

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Clinically, AGs are used to treat infections caused by aerobic Gram-negative bacilli as well as Gram-positive staphylococci, mycobacteria, some streptococci and others. Because of their structural differences, individual AG compounds differ in their effectiveness towards the various types of bacterial infections. Furthermore, AGs are often used in combination with other antibiotics, especially β-lactams or vancomycin, with which they work synergistically due to enhanced uptake of the AG. STR, the first drug discovered to be effective against TB, is still used, but less often due to high rates of resistance [9]. As a second line of defense, kanamycin A (KAN A) and amikacin (AMK) are used to treat multidrug-resistant (MDR)-TB infections, which are resistant to the front-line drugs isoniazid, rifampicin, and the fluoroquinolones. Also, AGs are used to treat life-threatening infections caused by enterococci and streptococci, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Yersinia pestis* (plague) and others. Newer AGs, such as AMK and arbekacin (ARB) are used to treat gentamicin (GEN)-resistant infections including methicillin-resistant *Staphylococcus aureus* (MRSA) [3]. Aside from being used as antibacterials, AGs have been explored for the treatment of genetic disorders featuring premature stop codons, such as cystic fibrosis and Duchenne muscular dystrophy [10], as well as in the treatment of Ménière's disease [11]. AGs are also being explored as HIV therapies as recently reviewed [2].

Clinical resistance to AG antibiotics is becoming a global health crisis as AGs are often second line or last resort treatments for the aforementioned deadly diseases including MDR-TB and MRSA infections. Bacterial resistance to an antibiotic arises from modification of the antibiotic target, efflux of the antibiotic or enzymatic modification of the antibiotic [12]. The most common mechanism of resistance to AGs is chemical modification by a family of enzymes called aminoglycoside-modifying enzymes (AMEs) [12]. There are three different types of AMEs: AG acetyltransferases (A ACs), AG nucleotidyltransferases (ANTs) and AG phosphotransferases (APHs). In Gram-positive pathogens, APH(3′)-IIIa and A AC(6′)-Ie/APH(2″)-Ia are two of the most common resistance enzymes [13]. Also, the prevalence of A AC(6′)-Ii in *Enterococcus faecium* leads to resistance to multiple AGs [14]. A multi-acetylating AME in *Mycobacterium tuberculosis*, the enhanced intracellular survival (Eis) protein is responsible for KAN A and in some instances AMK resistance in a significant fraction of KAN A-resistant clinical isolates of *M. tuberculosis* [15–21].

AACs use AcCoA as a cosubstrate. A ACs belong to the GCN5-related *N*-acetyltransferase superfamily of protein folds, which catalyze acetylation of free amines on the substrate molecule. Over 50 AACs have been identified in various organisms and they are capable of acetylation at the 1, 3, 2′ or 6′ positions of various AGs (Figure 1A). Eis is a unique enzyme capable of acetylating multiple positions on any given AG scaffold [22] and on lysine-containing molecules [23]. Eis homologues are found in a variety of mycobacteria (e.g., *Mycobacterium smegmatis* [24,25]) and non-mycobacteria (e.g., *Anabaena variabilis* [26]). There are five classes of ANTs, capable of adenylating at the 6, 9, 4′, 2″ or 3″ positions of AGs. APHs catalyze the transfer of a phosphate to the 4, 6, 9, 3′, 2″, 3″ or 7″ positions of AGs. ANTs and APHs both use ATP as a cosubstrate; ANTs transfer an adenosine monophosphate group to the AG substrate, while APHs transfer a single phosphate to the AG substrate. ANTs and APHs can also use GTP as a cosubstrate [27–30].

Traditionally, AMEs are named based on the AG position that they modify. For example, AAC(6′) acetylates at the 6′ position. Furthermore, a roman numeral, followed by a lower-case letter as an individual identifier describes AMEs based on their particular pattern of resistance. Ramirez and Tolmasky provide a comprehensive summary of the many AMEs identified [31]. Most AMEs are regioselective for a single position, though some are capable of multiple modifications. To date, four bifunctional AMEs have been identified and

studied: ANT(3″)-Ii/A AC(6′)-IId [32,33]; A AC(6′)-30/ A AC(6′)-Ib′[34]; A AC(3)-Ib/A AC(6′)-Ib′ [35,36]; and AAC(6′)-Ie/APH(2″)-Ia [37–40].

Crystal structures of all three types of AMEs, including that of Eis, have been determined, providing valuable structural information for efforts to thwart the AME modifications of AGs responsible for clinical resistance. All currently published AME structures are summarized in Tables 1–3. Many structures of AACs and APHs exist, while, to date, only three structures of ANT are available from the PDB. Structures of a sample of each type of AME are reported in Figures 2–4. In addition to these structures, a model of the bifunctional AME AAC(6′)-Ie/APH(2″)-Ia has been reported [41].

Multiple approaches have been explored to combat bacterial AG resistance by AMEs (Figure 5). Here, recent methods to overcome the increasing threat of AG resistance will be highlighted, discussing advantages and disadvantages of these approaches.

New AGs

Perhaps the most obvious method to evade AG resistance is to create new AGs, known as neoglycosides. The goal of this approach is to design novel AGs, or modify existing AGs, that will effectively bind to the bacterial ribosome and inhibit protein synthesis, but will not fit into the active site of AMEs. This type of strategy has been successfully applied to the discovery of novel β-lactam and other second- and third-generation antibiotics. In this section, recent highlights on progress towards developing new AGs by traditional chemical synthesis and by newer biosynthetic and chemoenzymatic approaches will be summarized. For more details on development of new AGs, we direct the reader to other excellent review articles [42–46].

Chemical synthesis of neoglycosides

Since the discovery of STR, with the goal of improving antibacterial activity and alleviating bacterial resistance associated with the action of AMEs, numerous AG derivatives have been synthesized. With the plethora and growing number of crystal structures of AMEs and A-site RNA fragments in complex with AGs, fragment-based virtual screening [47] and rational design of new AGs have gained popularity in the last decade. The strategies used to develop neoglycosides include: the formation of AG dimers; conformationally constrained AGs; AGs that can evade the action of multiple AMEs; and self-regenerating AGs (Figure 5).

AG dimers—As dimerized AGs displayed promise in enhancing RNA binding [48], early neoglycosides examined for bacterial MIC values and inactivation by AMEs were 5-linked neamine (NEA) dimers (Figure 6A) [49]. The best NEA dimers with methylene bridges of 3–5-C in length proved to be poor substrates of AAC(6′)-Ii, APH(3′)-IIIa and AAC(6′)-Ie/APH(2″)-Ia. Because of their improved antibiotic activity, affinity for the rRNA A-site and inertness towards AMEs, a variety of other AG homo- and heterodimers have been synthesized (Figure 6B) [50–53]. However, none of these novel AG dimers have been evaluated for their potential to resist the action of AMEs. It will be interesting to see, in the future, how these novel AG dimers will fair against AMEs. In addition to AG dimers composed of two complete distinct AGs, AG hybrids containing some features of neomycin B (NEO) or paromomycin (PAR) with sisomicin (SIS) were synthesized by Tsuji palladium-catalyzed deoxygenation at the 3′ and 4′ positions (Figure 6C) [54]. These hybrids were found to display activity superior to that of NEO and to evade APH(3′) and ANT(4′) activity in *P. aeruginosa* and *S. aureus*, respectively.

Conformationally constrained AGs—Several groups have attempted to make conformationally restrained AG derivatives that selectively tightly bind to the A-site rRNA sequence, but bind poorly to AMEs. Rigidified NEO derivatives with methylene (1- or 3-C) linkers between the 2′-NH and 5″-C were designed to resemble the conformation of NEO bound to A-site RNA, but to differ from the conformation of NEO in the ANT(4′) active site (Figure 6D) [55–58]. These compounds displayed good antibacterial activity and, as desired, were poor substrates for *S. aureus* ANT(4′) and *M. tuberculosis* AAC(2′). By using a similar approach, PAR analogs tethered between the 6-hydroxy and 6‴-amino moieties were designed (Figure 6E) [59]. The 6 and 6‴ positions were selected based on the differences between the structure of PAR bound to a A-site rRNA fragment and NEO bound to APH(3′)-IIIa. Unfortunately, these compounds were poor inhibitors of the growth of *S. aureus* and *Escherichia coli*, suggesting that this particular conformational constraint interferes with A-site binding.

AGs that evade the action of multiple AMEs—A large number of chemical modifications have been made to AG antibiotics in an attempt to evade the action of AMEs. By rational design, multiple series of NEA derivatives were synthesized [60,61]. In the first series, 4-amino-2-hydroxybutyryl (AHB) was inserted at position 1 of NEA to mimic that of AMK known to impart resistance to modification by various AMEs, whereas aliphatic amines were added at position 6 to cover the distance between the oxygen at that position and the phosphate backbone of the A-site of rRNA (Figure 7A) [60]. The two best NEA derivatives in that series were found to have activities superior to those of the parent drug and to be poor substrates of APH(3′)-I and A AC(6′)-Ie/APH(2″)-Ia. A small library of *O*-alkylated NEA derivatives was also prepared and an analog bearing 6-, 3′-, and 4′-(2-naphthylmethylene) substituents was found to be the most potent against *S. aureus* strains expressing APH(3′), ANT(4′), and A AC(6′)-Ie/APH(2″)-Ia as well as *P. aeruginosa* and *E. coli* strains expressing AAC(6′)-IIa, AAC(6′)-IIb, and ANT(2″)-IIa (Figure 7B) [61].

Based on the fact that substituents attached at the 4′ and 6′ positions of NEA have little effect on its binding to RNA and modification by AMEs would deactivate a non-4′-modified drug, a series of 4′-modified K AN B derivatives was synthesized to alleviate AME activity while retaining binding to RNA (Figure 7C) [62]. These compounds inhibited the growth of drug-resistant bacteria such as *Klebsiella pneumoniae* expressing ANT(2″), *P. aeruginosa* expressing APH(3′)-IIb, MRSA expressing APH(3′), ANT(4′) and A AC(6′)-Ie/APH(2″)-Ia, as well as *Staphylococcus epidermidis* expressing APH(3′)-IIIa. Based on the premise that acylated AGs are sometimes more potent and less toxic than their non-acylated counterparts, AGs acylated at the 6′ position have been synthesized to study the mechanism of AAC(6′)-Ii [63]. A series of 6′- and 6‴-*N*-acylated tobramycin (TOB) and PAR analogs was also synthesized [64]. These compounds, especially the 6′-*N*-glycinyl-TOB (Figure 7D), were active against a variety of Gram-negative and -positive bacterial strains and resistant to the action of a number of recombinantly overexpressed AMEs. As TOB derivatives proved promising in alleviating resistance caused by AMEs, a variety of 6″-thioether TOB analogs with aliphatic and aromatic chains was next investigated (Figure 7E) [65]. The best compounds bearing C_{12} and C_{14} aliphatic chains displayed lower MIC values than the parent TOB against several Gram-positive and -negative pathogens and resisted modifications by a variety of AMEs.

2″-*O*-substituted analogs of PAR were also synthesized, evaluated for their antimicrobial activities against *E. coli* and *S. aureus*, and their binding modes to the ribosome were investigated by co-crystallization with an RNA fragment [66]. Two compounds prevented *S. aureus* infection in mice with full protection at 1.2 and 0.5 mg/kg, respectively (Figure 7F). Based on these promising results, 2″-*O*-substituted PAR analogs further derivatized by

addition of an AHB group at position 1 were prepared [67]. These compounds were very active against many resistant *E. coli*, *S. aureus* and *S. epidermidis* strains.

Finally, a series of 5-*epi*-substituted-4′-hydroxy derivatives of ARB was prepared and found to display increased activity against MRSA strains expressing A AC(6′)-Ie/APH(2″)-Ia (Figure 7G) [68]. The improved MIC for the 5-*epi*-substituted-4′-hydroxy derivative is likely due to its evasion of modification by ANT(4′). Additional 4″-*epi*- and 5-*epi*-derivatives of ARB also demonstrated activity against MRSA and *Pseudomonas* strains in which AMEs are expressed [69].

Self-regenerating AGs—By phosphorylating AGs at their 3′-hydroxyl, APH(3′)s decrease the binding aff inity of these AGs for the bacterial ribosome. A ‘self-regenerating’ K AN A derivative that avoids inactivation by APH(3′) enzymes was cleverly designed (Figure 8) [70]. This self-regenerating AG is inherently unstable and is hydrolyzed to the 3′-geminal diol, which was found to be a viable substrate for both APH(3′)-Ia and APH(3′)-IIa. After enzymatic modification, the phosphorylated product undergoes non-enzymatic elimination, regenerating the initial KAN A analog. This self-regenerating AG exemplifies the creativity that will be needed to evade the action of AMEs and overcome bacterial resistance.

Other AGs with antimicrobial potential—Some additional promising AG derivatives, but by no means an exhaustive list, are presented in this section. The effect of guanidinyl additions to various AG scaffolds was recently explored [71]. Using a fluorescence resonance energy transfer binding assay for the rRNA A-site, many of the guanidinylated AGs demonstrated increased affinities and lower MIC values against resistant strains when compared with the parent compound. AGs coupled to peptides and lipids were prepared with the intention of increasing bacterial uptake [72,73]. When examining TOB–lipid and –peptide conjugates, it was observed that the length of the lipid tail has a large effect on antibacterial activity, while the number of positive charges plays a lesser role [72]. NEO-C_{16} and -C_{20} lipid conjugates demonstrated strong activity against Gram-positive bacteria and antibacterial activity towards MRSA [74]. Guanidinylated NEO and KAN A lipid conjugates with C_{16} and C_{20} tails were found to restore anti-MRSA activity and overcome KAN A resistance [75]. Cationic AG polycarbamates and polyethers were synthesized and polyol-modified NEO exhibited enhanced antibacterial activity against resistant strains [76]. NEO– and KAN A–peptide triazole conjugates demonstrated better activity when compared with parent compounds against NEO- and KAN A-resistant bacterial strains, respectively [77].

Many AG derivatives were recently synthesized for purposes other than improving antibacterial activities. Some of these have undergone antimicrobial studies, but have not yet been evaluated for their ability to resist AMEs. AG conjugates have also been prepared as telomerase inhibitors [78]. Recently, AG derivatives were explored as potential therapeutics for genetic diseases caused by premature stop codons brought on by random mutations [10,79–81]. Though optimized to aid in read-through of premature termination codons at the eukaryotic ribosome, this armory of multiple generations of AGs can also be investigated for their alternative potential as antibacterial agents. Preliminary studies have demonstrated the promise of some of these compounds to evade the action of APH(3′)-IIIa. Overall, it will be interesting to observe how these new-generation AGs will fair against AMEs.

Plazomicin, the newest AG in clinical trials—Currently, the only neoglyoside that has advanced to clinical trials is plazomicin (PLZ), formerly known as ACHN-490 (Figure 9) [82,83]. PLZ is a synthetic derivative of SIS with an AHB substituent at position 1 and a hydroxyethyl substituent at the 6′ position [44]. While maintaining high binding affinity for

the bacterial ribosome, PLZ is resistant to the action of most AMEs (A AC(3), APH(3′), ANT(4′), A AC(6′), ANT(2″) and APH(2″)), with the exception of A AC(2′) enzymes to which it remains susceptible. PLZ displays broad-spectrum activity and is effective against both Gram-positive and -negative bacteria, including several AG-resistant strains expressing AMEs. PLZ has been found to be effective against many drug-resistant clinical isolates from the USA including: *Klebsiella pneumoniae* [84]; *E. coli* and *K. pneumoniae* [85]; MRSA [86]; as well as *Acinetobacter baumannii* and *P. aeruginosa* [87]. PLZ was also found to be effective against carbapenem-resistent *Enterobacteriaceae* from the UK [88] as well as AG-resistant strains of *K. pneumoniae*, *E. coli* and *Enterobacter* spp. from Greece [89]. Furthermore PLZ was effective against resistant *E. coli*, *Enterbacteriaceae* and MRSA infections in mouse models [90]. Against *P. aeruginosa*, PLZ was found to be synergistic when used in combination with other drugs including cefepime, doripenem, imipenem and piperacillin/tazobactam [91]. In another study, PLZ in combination with daptomycin, both at sub-inhibitory concentrations, were found to act synergistically against over 40 MRSA bacterial strains [92]. Phase I clinical trials performed by Achaogen Inc. have been successful and revealed no evidence of ototoxicity or nephrotoxicity for PLZ [93]. Phase II clinical trials for patients with complicated urinary tract infections were successfully completed in April 2012. Additional trials are planned for 2013.

Biosynthesis & chemoenzymatic formation of new AG derivatives

To circumvent the problems associated with the complexity of traditional chemical approaches for the development of AGs, biosynthetic and chemoenzymatic methodologies have been developed. Rhodostreptomycin A and B were biosynthetically produced by horizontal gene transfer from *Streptomyces padanus* to *Rhodoccoccus fascians* (Figure 10A) [94]. Rhodostreptomycin was tested against several bacterial strains, but has yet to be evaluated in regards to resistant strains and AMEs. However, it sets precedence for exploiting bacterial gene transfer [95,96], an advantageous method that alleviate the synthetic challenges presented by complex AG scaffolds, for the production of new AG derivatives.

By using biosynthetic enzymes from *Bacillus circulans*, BtrH and BtrG, to install an AHB moiety at position 1 of NEO and K AN A, the AGs neokacin and AMK were generated, respectively (Figure 10B) [97]. This method is valuable as it can be applied to milligram-scale **chemoenzymatic synthesis** of unnatural AHB-substituted AGs. By taking advantage of the cosubstrate promiscuity of AACs, the resistance enzymes themselves, novel mono- as well as homo- and hetero-di-*N*-acylated AGs were prepared (Figure 10C) [98]. While only small amounts of AG derivatives can be produced from these chemoenzymatic reactions, this method presents an excellent initial screen to determine which compounds warrant further investigation and the pursuit of a larger-scale synthesis.

AME inhibitors

Since the majority of antibiotic resistance arises from the action of AMEs, the design of AME inhibitors is another promising strategy to evade resistance. This approach has been effectively applied to β-lactams: the combination of β-lactams antibiotics and inhibitors of β-lactam resistance enzymes (β-lactamases) has been clinically and commercially successful for over 30 years [99]. Similarly, AME inhibitors could become **adjuvant** antibiotics; their co-delivery along with an AG would prevent inactivation of the AG by the resistance enzymes. AGs are already often dosed in combination with other antibiotics; AME inhibitors would be one more compound to add to the cocktail of drugs.

The first efforts towards developing AME inhibitors began over three decades ago. Shortly after the identification of AcCoA as the cosubstrate of AACs, a derivative of GEN with

AcCoA attached at the 3-NH_2, termed a 'multi-substrate', was prepared chemoenzymatically and evaluated as an AAC(3′) inhibitor [100]. In this section, recent highlights on progress towards developing AME inhibitors will be summarized.

Bisubstrate (AG-AcCoA) inhibitors targeting AAC(6′)

AG–AcCoA bisubstrate inhibitors of AAC(6′) were designed as potential drug candidates and for mechanistic studies (Figure 11A) [43]. NEA-AcCoA with linkers of varying length (1–5C) proved to be good inhibitors of A AC(6′)-Ii, A AC(6′)-Iy, and A AC(6′)-Ie/APH(2″)-Ia, with nanomolar to micromolar potencies [101]. These molecules were crystallized with A AC(6′)-Ii, providing valuable structural information regarding the active site of the enzyme [102]. Bisubstrate inhibitors with sulfonamide- and phosphonate-containing linkers to mimic the proposed tetrahedral transition-state geometry were synthesized, but were found to not increase in potency [103]. Two similar compounds made with sulfone linkers had nanomolar potency for AAC(6′)-Ii, suggesting that the S=O was correctly positioned to hydrogen bond to nearby Tyr111 or Tyr147 hydroxyls [103,104]. Bisubstrate analogs with K AN A or RIB in place of NEA displayed similar potencies to the NEA derivatives, suggesting that the AG rings III and IV are not necessary for inhibition [101]. An analog with only ring I loses potency, indicating the importance of ring II in binding [105].

Although they are potent inhibitors that have revealed much regarding the active site and the mechanism of AAC(6′), bisubstrate compounds were not effective in cellular assays, probably due to poor membrane permeability because of their large size and negative charge. To address this, the anionic adenosine diphosphate moiety, was modified. SAR studies revealed that the adenosine is not essential, but potency drops dramatically if more than one phosphate is removed [105]. Crystal structures demonstrate several hydrogen bond donors positioned to interact with the negatively charged phosphates, supporting their importance in potent inhibitor binding [43]. Recently, another attempt to overcome poor cell membrane permeability includes the design of bisubstrate prodrugs consisting of only an AG with a pantetheine linker (Figure 11B) [106]. This linker was chosen to take advantage of bacterial enzymes PanK, PPAT and DPCK, which, once inside the cell, transform the AG–pantetheine into the desired AG–pantetheine–CoA compounds. These compounds were also tested against an AG-resistant strain of *Enterococcus faecium* that expresses AAC(6′)-Ii and were found to be effective inhibitors of bacterial growth when co-delivered with KAN A.

A bisubstrate scaffold has inspired other efforts towards AME inhibitors. Using NMR-guided fragment-based design, bisubstrate-like inhibitors in which the AG is replaced with a mimicking fragment were designed and had nanomolar inhibition of AAC(6′)-Ib (Figure 11C) [107].

APH inhibitors

Diverging from the conventional small-molecule chemotherapeutic approach, *in vitro* and *in vivo* inhibition of APH(3′)-IIIa was achieved by a designed ankyrin repeat (AR) protein [108]. The 33-amino acid peptide was selected from a library of designed AR proteins. The larger surface area of a small protein enables it to bind its target with higher specificity than small molecules, however, therapeutic delivery of proteins is often more complicated than small molecules. Crystal structures of APH(3′)-IIIa in complex with the AR inhibitor revealed the allosteric binding mode of the AR inhibitor, which is responsible for rendering the APH inactive [109].

Because the APHs possess kinase activity, previously discovered eukaryotic protein kinase inhibitors have been investigated as APH inhibitors. Early work identified the known kinase

inhibitors quercetin and CKI-7 (Figure 12A), among others, to be inhibitors of APH(3′)-IIIa and A AC(6′)-Ie/A PH(2″)-Ia [110]. More recently, CKI-7, an ATP-competitive inhibitor for casein kinase 1, was further studied as an inhibitor of APH(3′)-IIIa and APH(9)-Ia [111]. Co-crystal structures of both of these APHs with CKI-7 were solved, revealing that the binding mode of the inhibitor in the nucleotide-binding pocket of these bacterial enzymes is indeed different from that of CKI-7 with eukaryotic kinases. This allows for potential development of bacterial kinase inhibitors selectively targeting APHs.

Fourteen APHs were recently screened against a commercially available library of 80 chemically diverse kinase inhibitors to map the resistance kinase chemical space [112]. The screens identified molecules with both broad and narrow inhibition profiles, suggesting that several protein kinase inhibitors warrant further studies as effective AME inhibitors. One hit was the aforementioned natural product kinase inhibitor quercetin (Figure 12A), which inhibited several of the APHs *in vitro* and *in vivo*. The co-crystal structures of APH(2″)-IVa in complex with quercetin, as well as with KAN A, were solved, providing important structural information to further the design of APH inhibitors.

Relevant to future APH inhibitor design, it was discovered that while APH(3′)-IIIa exclusively uses ATP as a cosubstrate, APH(2″)-Ib is capable of using both ATP and GTP [28]. GTP use among human kinases rarely occurs, hence these differences may be exploited for kinase inhibitors that selectively target APHs without disrupting human kinases.

Inhibitors that target multiple AMEs

Even though APH inhibitors are useful, compounds that inhibit multiple AMEs would be desirable. A library of 45 non-carbohydrate AME inhibitors were designed based on the 1,3-diamine pharmacophore found in AG structures and were observed to be competitive inhibitors of ANT(2″)-Ia and APH(3′)-IIIa (Figure 12B) [113]. A structure–activity relationship of this small group revealed that the 3-(dimethylamino)propylamine moiety was common in the most potent inhibitors.

The previously described set of 2″-ether PAR analogs (Figure 7F) [66] were later investigated as inhibitors of APH(3′)-IIIa and AAC(6′)-Ii [114]. Of the 25 compounds reported, all were poorer substrates for AAC(3′)-IIIa than PAR and four had no detectable turnover with this AME. The 2″-substituted PAR analogs had low mircomolar K_i values for the two AMEs examined. This study is a great example of the collaboration and resourcefulness (evaluating existing compounds for multiple purposes) needed to tackle the problem of bacterial resistance.

Natural products

Aranorosin (Figure 12C), a natural product isolated from *Gymnascella aurantiaca*, inhibited the growth of a MRSA strain only in the presence of ARB, and was confirmed to be acting by inhibiting the bifunctional AME AAC(6′)-Ie/APH(2″)-Ia [115]. Four biverlactone natural products isolated from *Penicillium* sp. FKI-4429 that circumvent ARB resistance in MRSA cultures have also been identified, however, their mechanisms of action have not yet been characterized [116]. Hopefully more of the thousands of natural products already isolated and characterized by scientists will be screened as AME inhibitors in the future.

Cationic peptide inhibitors of AMEs

Several cationic peptides that mimic the binding of AGs to the negatively charged active site of AMEs have been examined. These peptides have good affinity for AAC(6′)-Ie, AAC(6′)-Ii, APH(3′)-IIIa and A AC(6′)-Ie/APH(2″)-Ia, and are the first compounds discovered to demonstrate broad-spectrum inhibition of AMEs. However, these peptide inhibitors did not

demonstrate any antimicrobial effects against resistant bacterial strains, probably due to poor membrane permeability [117].

High-throughput screens for AME inhibitors

Well-established enzyme assays that can be adapted to high-throughput screening (HTS) exist for all three classes of AMEs, making HTS a realistic approach to finding AME inhibitors. Surprisingly, HTS has so far only been applied to the identification of inhibitors of Eis, the multi-acetylating A AC from *M. tuberculosis* [118]. From a library of approximately 23,000 compounds, 25 compounds were identified with activities in the high nanomolar to low micromolar range. While they vary in structure, these 25 compounds all feature at least one aromatic ring and one amine functional group. Some compounds demonstrated mixed inhibition, while others were found to be competitive inhibitors against AGs. It will be interesting to see how these compounds fair in restoring the activity of KAN A in extensively drug-resistant TB clinical isolates known to resist this AG. Crystal structures of Eis–AcCoA–inhibitor complexes could also provide valuable information for further development of inhibitors of this AME. HTS appears to be a promising strategy to discover new compounds for drug combinations with currently approved AGs for TB treatment. As several AMEs exist, this strategy could be potentially applied to discover inhibitors of other AMEs for the treatment of resistant bacterial pathogens besides TB. Even though no screens for inhibitors of APHs have been reported, thus far, as the many HTS for other kinases have already been successful [119,120], there is high hope that novel APH inhibitors could be identified this way. While the co-delivery of existing AGs with new AME inhibitors is a seemingly straightforward approach because AMEs are ‘drug gable’ enzymes, it is important to note that this solution would not eliminate the toxicity of the currently used AGs.

High-throughput methods to evaluate new AGs & new AME inhibitors

As the number of AG derivatives and potential AME inhibitors keeps growing, it is becoming pertinent to develop efficient high-throughput methods to assess the potential of these compounds to meet the various criteria required of them to become successful antibacterials. To be good antibiotics, new AGs must: bind to the prokaryotic (but not the eukaryotic) ribosome; not be a substrate for AMEs; and demonstrate bacterial growth inhibition. AME inhibitors must inhibit one or ideally many AMEs without disrupting binding of AGs to the ribosome.

Screens for ribosome binding

While AG binding affinity to the ribosome is not directly proportional to MIC, it is often a useful means of identifying potential AGs [121]. An early HTS employed a competitive fluorescent (pyrene-labeled) AG probe (PAR or TOB) and a prokaryotic ribosomal RNA A-site construct known to bind AGs [122]. Monitoring fluorescence quenching measured antagonist binding to the rRNA fragment. This study served as a launching pad for the development of other AG-fluorescent probes and future HTS for RNA-binding compounds. These ribosomal binding affinity screens are additionally useful as they may be adapted to use a eukaryotic RNA A-site fragment to identify compounds with predictably fewer toxic side effects [123]. Earlier this year, a related high-throughput assay that uses a robust and reproducible fluorescein-conjugated NEO compound as a competitive binding probe was developed to measure binding affinity of a compound to an *E. coli* RNA A-site fragment [124]. This proof-of-principle study screened existing parent AGs (NEO, GEN, PAR, NEA, RIB and STR) and proved to be a promising tool to identify new high-affinity ribosome-binding compounds.

HTS using methods other than fluorescence-based probes, namely MS [125,126], NMR [127] and surface plasmon resonance [128] have also been developed to measure binding affinity for the RNA A-site. Isis Pharmaceuticals has developed a MS assay, which, in addition to determining binding affinities of AGs for RNAs, also identified binding specificity based on the fragmentation pattern of AG/RNA complexes [126]. This assay has been used to assess the binding affinities of a library of heterocyclic PAR analogs, identifying a derivative with good RNA-binding affinity, more potent than TOB and APR, but not quite as potent as PAR itself [129]. In 2003, Abbott reported a NMR-based screen to identify compounds that bind to the *E. coli* ribosomal A-site [127]. This screen of approximately 10,000 compounds resulted in initial hits (hit rate 3%) with binding affinities from 70 μM to 3 mM. HTS, such as those described above, should help in identifying novel AGs that efficiently bind to their target.

High-throughput microarrays

A high-throughput microarray platform for directly assaying the activity of resistance enzymes on AGs and the effect of immobilization on RNA binding has been developed (Figure 13) [130,131]. This method relies on arrays of AGs immobilized by an azido attachment at their 6″ position and monitors the reactivity of AGs with AMEs by radioactivity and their binding to the A-site of rRNA by fluorescence. Thus far, this method has been tested with KAN A and TOB derivatives against ANT(2″) and APH(3′). It has also served as a basis for a 2D combinatorial screen of small RNA internal loops to determine their binding affinities for the immobilized AGs [132]. Even though the method is optimized and relatively straightforward, it does require the use of specialty equipment and radioactivity as well as challenging synthesis of 6″-azido-AGs.

Regulating AME expression

An alternative approach to overcome the action of AMEs consists of blocking the expression of genes encoding for these resistance enzymes. Antisense oligodeoxynucleotides were first designed to block expression of the *aac(6′)-Ib* [133]. Although successful, this method was limited by the fact that it required electroporation to introduce the oligonucleotide into the bacterial cells. Subsequently, short antisense oligonucleotides that induce cleavage of mRNA, termed external guide sequences, were designed to induce inhibition of AMK resistance caused by A AC(6′)-Ib [134]. While successful, the external guide sequences olignonucleotides were rapidly degraded by nucleases *in vivo*. To circumvent this problem, locked nucleic acid (LNA)/DNA co-oligomers resistant to nucleases were next investigated [135]. The most successful, ‘LNA9’, inhibited growth of *E. coli* AS19 expressing AAC(6′)-Ib at a concentration of 50 nM when co-delivered with AMK and was stable to nucleases for over 24 h. Synthetic RNA silencing has been successfully applied to a variety of other bacterial resistance genes, but not to other AMEs yet [136].

Drug combinations & repurposing

Many antibiotics are already commonly used in combination therapies to broaden their antimicrobial spectrum and generate synergistic effects by hitting more than one target. Drug combinations may also help to combat antibiotic resistance arising from the expression of AMEs. Furthermore, the repurposing of drugs is advantageous because molecules developed to the point of clinical trials will have well-characterized pharmacology and toxicology. Redeployment of existing molecules is also economical with regards to both the time and cost required by the early stages of the drug-discovery process. As previously mentioned, the repurposing of chemical libraries of kinase inhibitors, developed for campaigns in cancer and other diseases involving protein kinases, can identify molecules with orthogonal ability to inhibit antibiotic resistance kinases. Such compounds could be

formulated as co-drugs to overcome antibiotic resistance. Both repurposing and drug combinations should be part of the solution to the relentless problem that is antibiotic resistance.

Many examples of drug combinations have been explored to fight bacterial infections [137]. A recent highlight includes the use of two β-lactams to effectively treat extensively drug-resistant TB [138]. Also, TOB and the macrolide antibiotic clarithromycin have demonstrated promising synergistic effects in *M. tuberculosis* clinical isolates [139]. Recent work demonstrates that, in general, combinations of antibiotics and non-antibiotic drugs could result in enhancement of antimicrobial activity [140]. Initially, a library of over 1000 previously approved drugs were screened to identify compounds that augment the activity of minocycline, a tetracycline antibiotic that inhibits protein synthesis. The combinations were assayed against strains of *P. aeruginosa*, *E. coli*, and *S. aureus*. A total of 69 non-antibiotic compounds exhibited synergistic activity against these pathogens when combined with minocycline. These compounds were then tested, again in combination, against a panel of clinical MDR strains. Some compounds were selective, inhibiting the growth of only a few of the strains, while a few, including loperamide, an opioid receptor agonist used to treat diarrhea, were synergistic with multiple antibiotics, including AGs.

An emerging branch of combination therapies is dual-acting (or hybrid) antibiotic molecules [141]. These heterodimeric molecules contain two individual antibiotic moieties, which maintain different targets, covalently linked together. The covalent linker may be a cleavable, in which case the hybrid molecule is a prodrug, or the linker may be stable, in which case the hybrid molecule is a dual-acting drug. A recent series of dual-acting antibiotics contained ciprofloxacin, a fluoroquinolone antibiotic, covalently linked to NEO [79]. Many of these compounds were more potent than NEO alone against several NEO-resistant bacterial strains, and were inert to modification by APH(3′)-Ia, APH(3′)-IIIa, and AAC(6′)-Ie/ APH(2″)-Ia. Notably, in multi-passage experiments the dual-acting ciprofloxacin–NEO compounds demonstrated a significant delay in the development of resistance in both Gram-negative and -positive bacterial strains.

Future perspective

This article summarizes the many various efforts underway to counteract bacterial resistance caused by AMEs. A combination of many of these highlighted strategies, as well as new, creative methods will likely be necessary to suppress resistance. It will take a combination of efforts from the health-care sector, academic and private research, as well as the community at large to recognize the global need for new antibiotics and take responsible actions when using antibiotics.

The tried and true method of designing new AG derivatives has been fruitful in the past, resulting in successes, such as ARB and AMK. Although still challenging, recent improvements in synthetic, biosynthetic, and chemoenzymatic methodologies render the discovery and study of neoglycosides more feasible. As new AGs are pursued, it should be taken into account that many existing AGs are ototoxic, therefore, scaffolds that have demonstrated less toxicity, such as APR, should probably be investigated as parent compounds. New technologies should be utilized including innovative synthetic approaches and HTS for activity checks with AMEs and for measuring rRNA A-site binding. As natural products, AG biosynthetic pathways could be exploited to produce new AGs [4]. However, as history demonstrates, resistance to new AGs is inevitable. As with all next-generation antibiotics, it will be a never-ending struggle to stay one step ahead of resistance.

In the future, scientists will likely move away from traditional medicinal chemistry and towards more modern methods, such as drug repurposing. Currently, there are many drugs

that are already in use that have been well studied in terms of their dosing, metabolism and toxicology. Repurposing these existing compounds, already evaluated to be safe, as AME inhibitors will require many fewer man-hours than *de novo* designs. Of course, resistance to these inhibitors will almost certainly arise in the future and, therefore, it is not to be forgotten that we may never overcome antibiotic resistance.

Key Terms

Chemoenzymatic synthesis	Use of enzymes to aid in the synthesis of organic compounds
Adjuvant	Secondary compound that modifies, usually positively, the effect of the primary compound in a combination therapy

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Executive summary

Background

- Many methods are being explored to address the crucial and urgent need to overcome bacterial resistance to aminoglycoside (AG) antibiotics caused by aminoglycoside-modifying enzymes (AMEs).

Summary of strategies of overcome the action of AMEs

- Existing AG scaffolds can be modified to create new AGs capable of evading AME modification. Future focus should be put on those associated with the least resistance and toxicity.
- AME inhibitors can be designed to be co-delivered along with current AGs.
- Innovative chemoenzymatic and biosynthetic approaches will expand the repertoire of accessible scaffolds and facilitate synthesis of novel AGs and AME inhibitors, which often contain multiple stereocenters.
- Repurposing existing drugs offers an economically advantageous drug-discovery approach that should be applied to overcome AME resistance. For example, previously identified protein kinase inhibitors offer chemical scaffolds that can block antibiotic resistance caused by AG phosphotransferases, providing leads for drug combination.

Future perspective

- Resistance results from a natural evolutionary phenomenon that, in all probability, will never end. Appropriate use of existing AGs and the continual development of new AGs as well as novel antibiotic adjuvants using a variety of the strategies described in this review will hopefully sustain minimal levels of resistance.

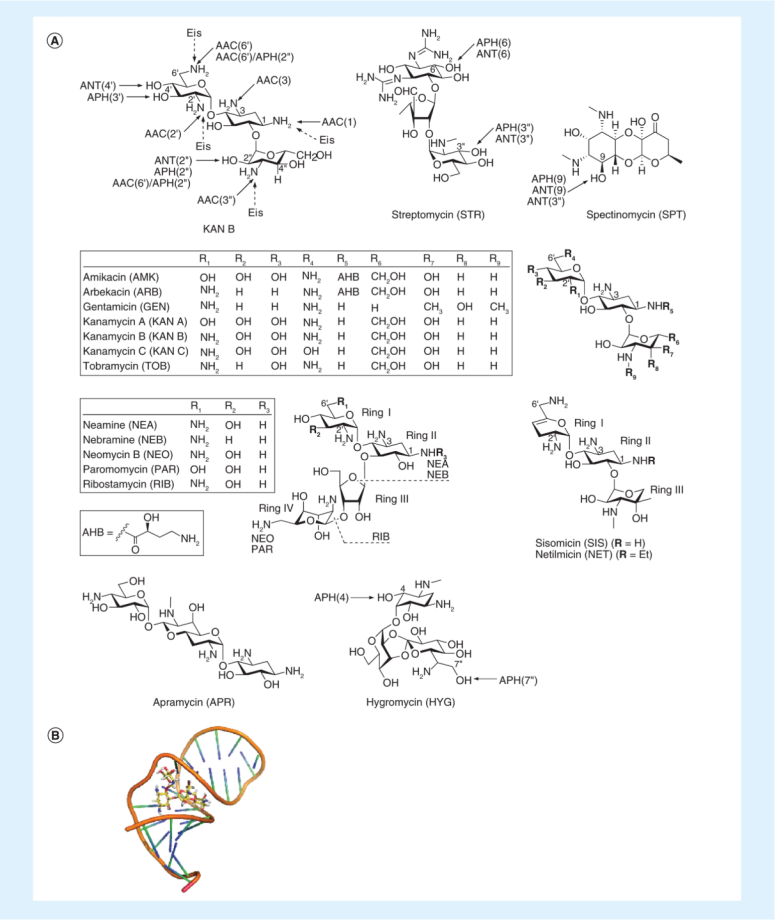


Figure 1. Aminoglycosides

(A) Aminoglycoside antibiotics with summary of positions modified by aminoglycoside-modifying enzymes (indicated by solid line arrows on representative structures of kanamycin B, streptomycin, hygromycin and spectinomycin). The dashed arrows indicate potential sites of modifications by the multi-acetylating aminoglycoside-modifying enzyme enhanced intracellular survival protein. **(B)** 16S rRNA in complex with paromomycin (PDB code: 1PBR [142]).

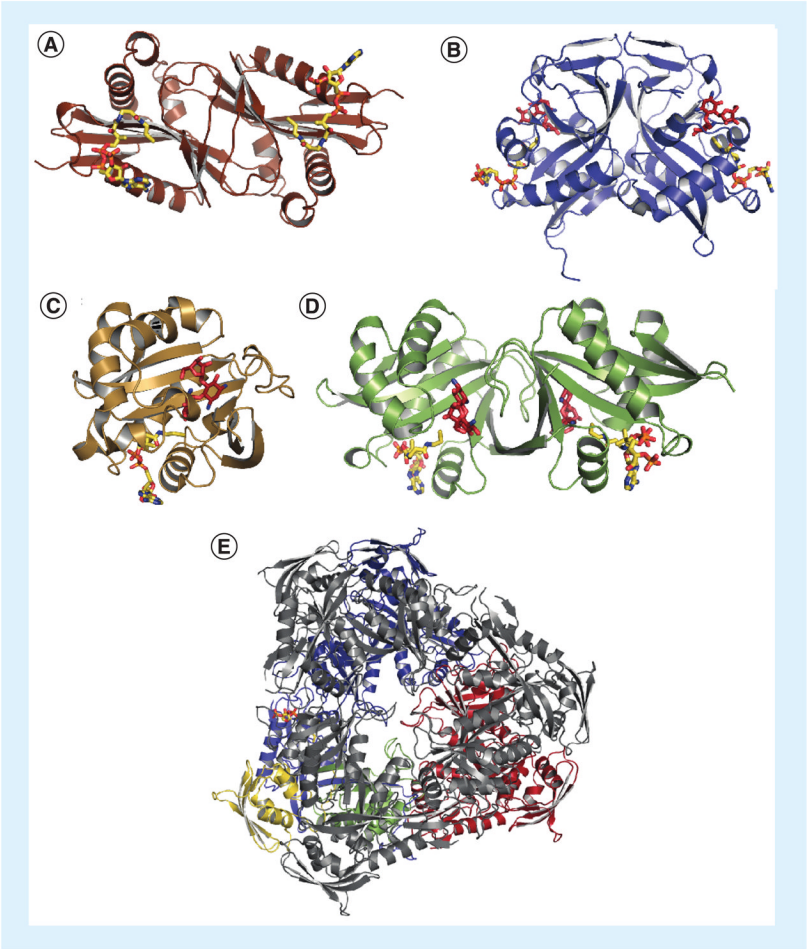


Figure 2. Representative aminoglycoside acetyltransferases (AACs)

(A) AAC(3)-Ia with CoA (sticks) (PDB code: 1BO4) [143]. **(B)** AAC(2′)-Ic with RIB (ribostamycin; red sticks) and CoA (yellow sticks) (PDB code: 1M4G) [145]. **(C)** AAC(6′)-Ib with RIB (red sticks) and CoA (yellow sticks) (PDB code: 2BUE) [146]. **(D)** AAC(6′)-Iy with RIB (red sticks) and CoA (yellow sticks) (PDB code: 1S3Z) [150]. **(E)** Enhanced intracellular survival protein with CoA (sticks (PDB code: 3R1K) [22].

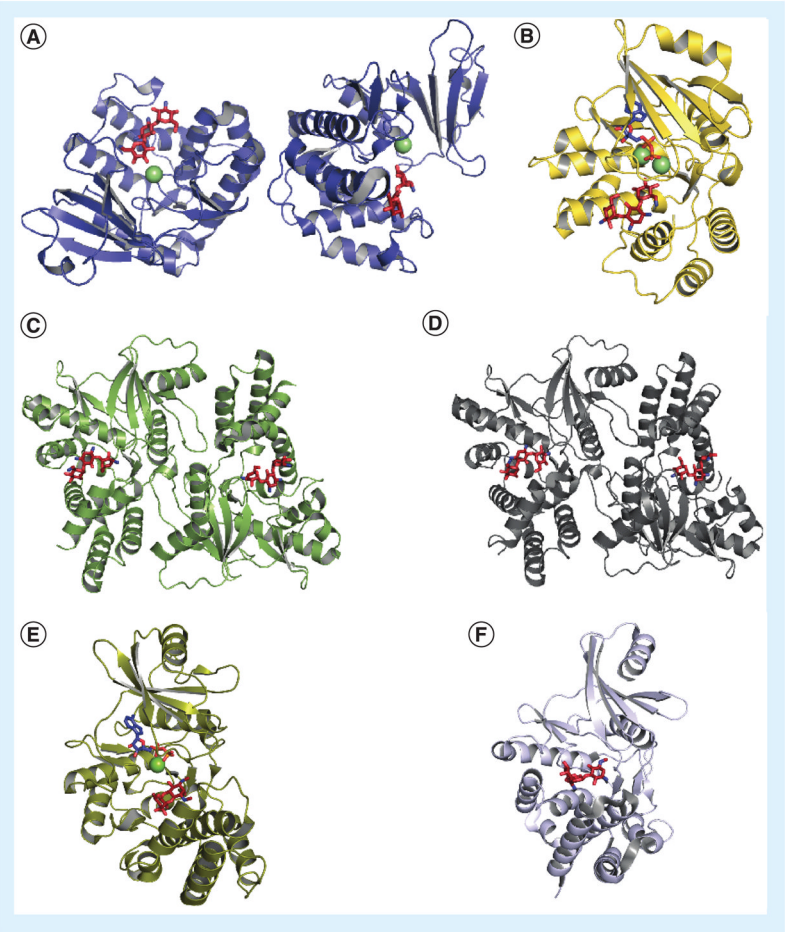


Figure 3. Representative aminoglycoside phosphotransferases (APHs)

(A) APH(3′)-IIa with KAN A (kanamycin A; sticks) and Mg^{2+} ions (spheres) (PDB code: 1ND4) [151]. **(B)** APH(3′)-IIIa with KAN A (sticks) and Mg^{2+} ions (spheres) (PDB code: 1L8T) [153]. **(C)** APH(2″)-IVa with KAN A (sticks) (PDB code: 3SG9) [157]. **(D)** APH(2″)-Id/APH(2″)-IVa with KAN A (sticks) (PDB code: 4DFB) [112]. **(E)** APH(9)-Ia with SPT (red sticks), ADP (blue sticks), and Mg^{2+} ions (spheres) (PDB code: 3I0O) [159]. **(F)** APH(4)-Ia with hygromycin (sticks) (PDB code: 3TYK) [158].

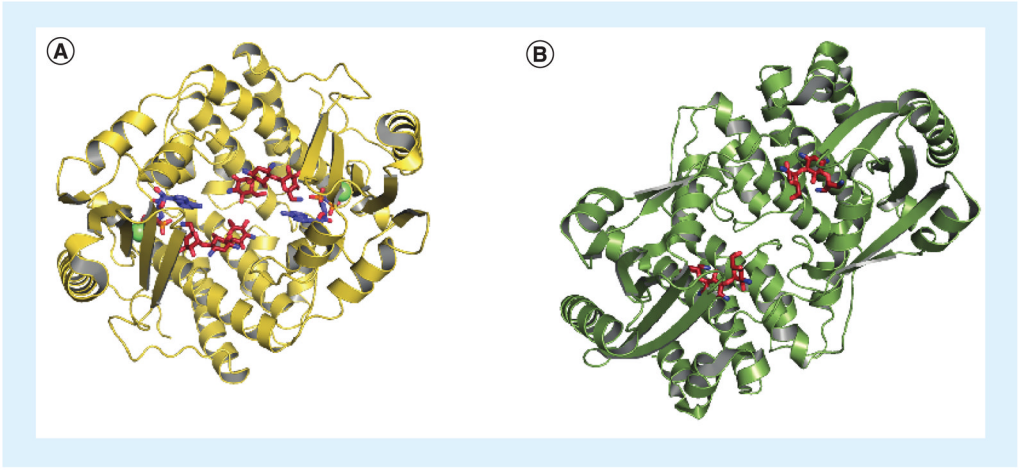


Figure 4. Representative aminoglycoside nucleotidyltransferases (ANTs)

(A) ANT(4′) with kanamycin A (red sticks), adenosine 5′-(β,γ-imido)triphosphate (blue sticks), and Mg^{2+} ions (spheres; PDB code: 1KNY) [160]. **(B)** ANT(4′)-IIb with tobramycin (sticks; PDB code: 4EBK).

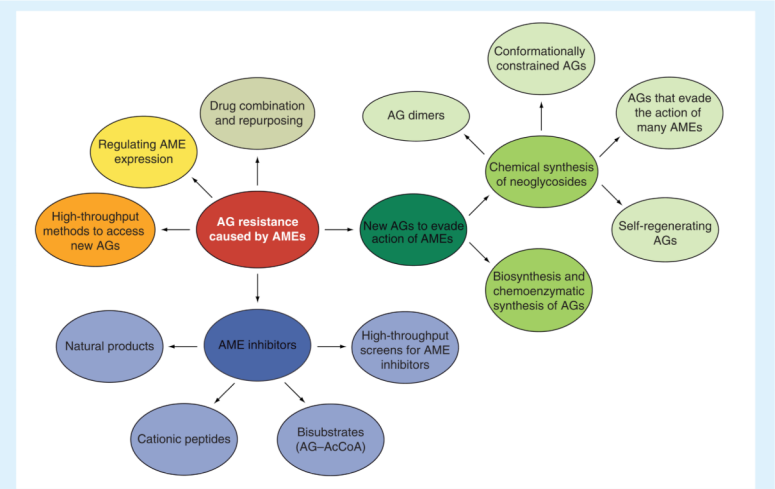
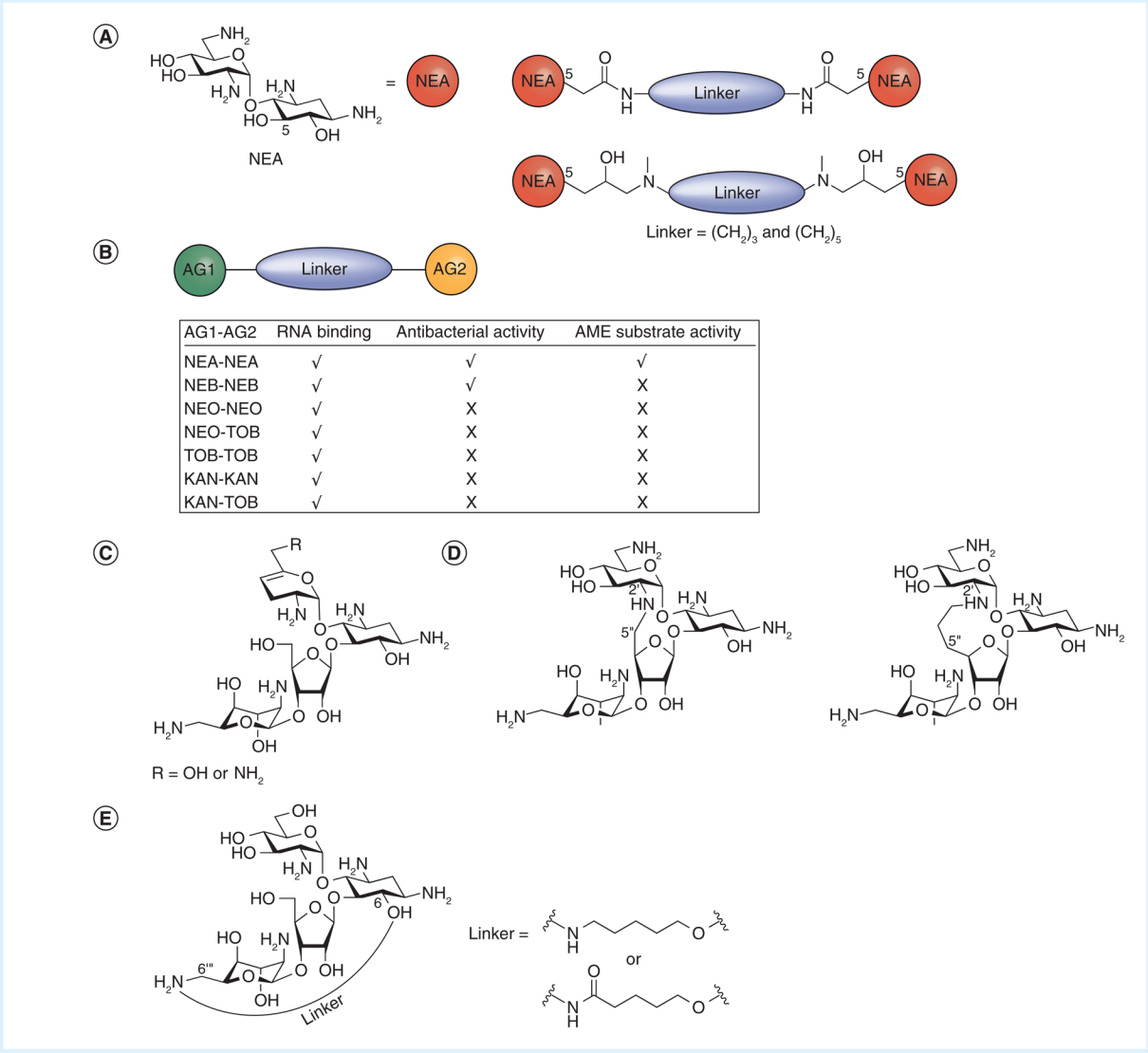


Figure 5. Overview of strategies to overcome resistance to aminoglycoside-modifying enzymes discussed in this review

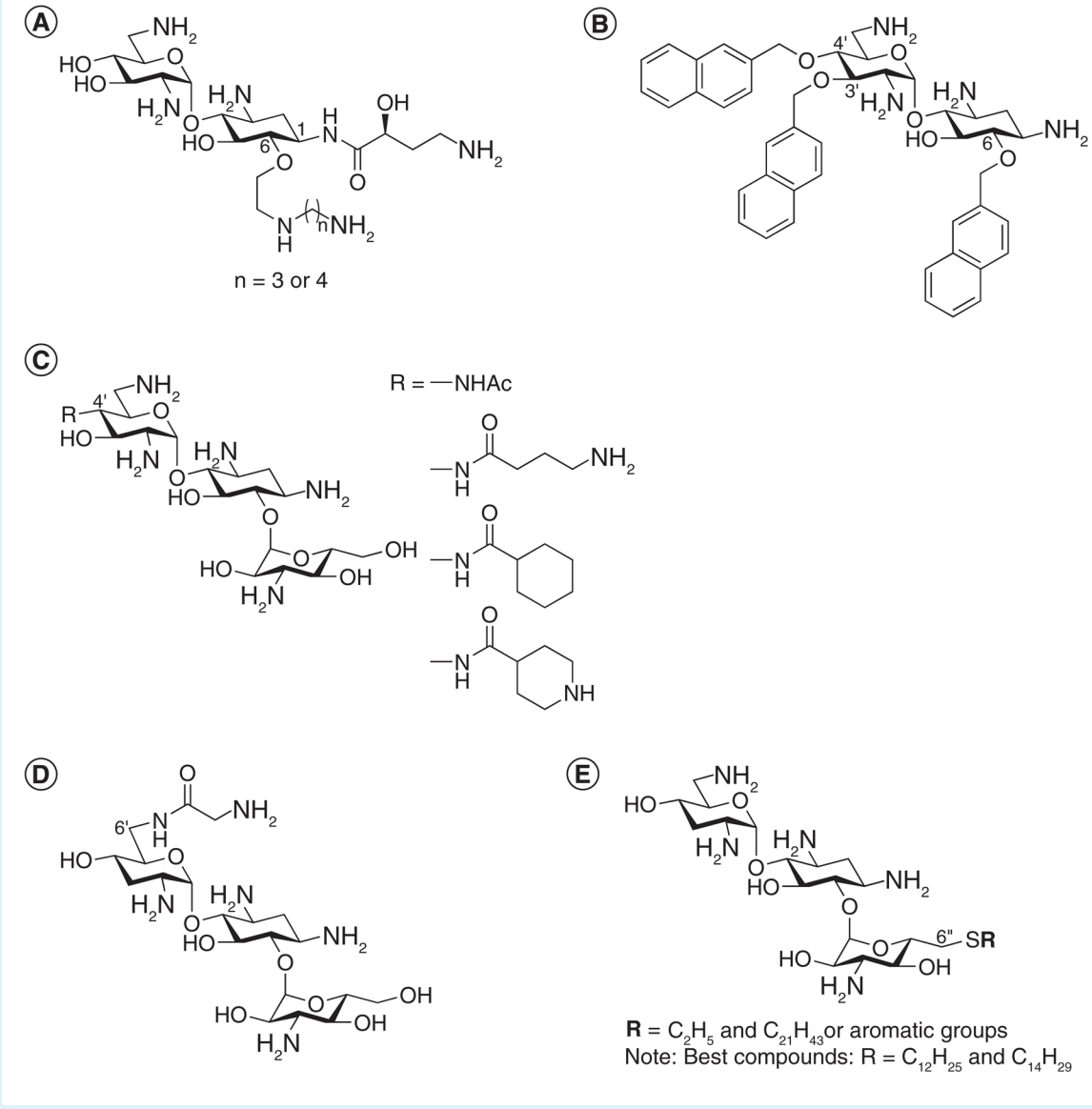
AG: Aminoglycoside; AME: Aminoglycoside-modifying enzyme.

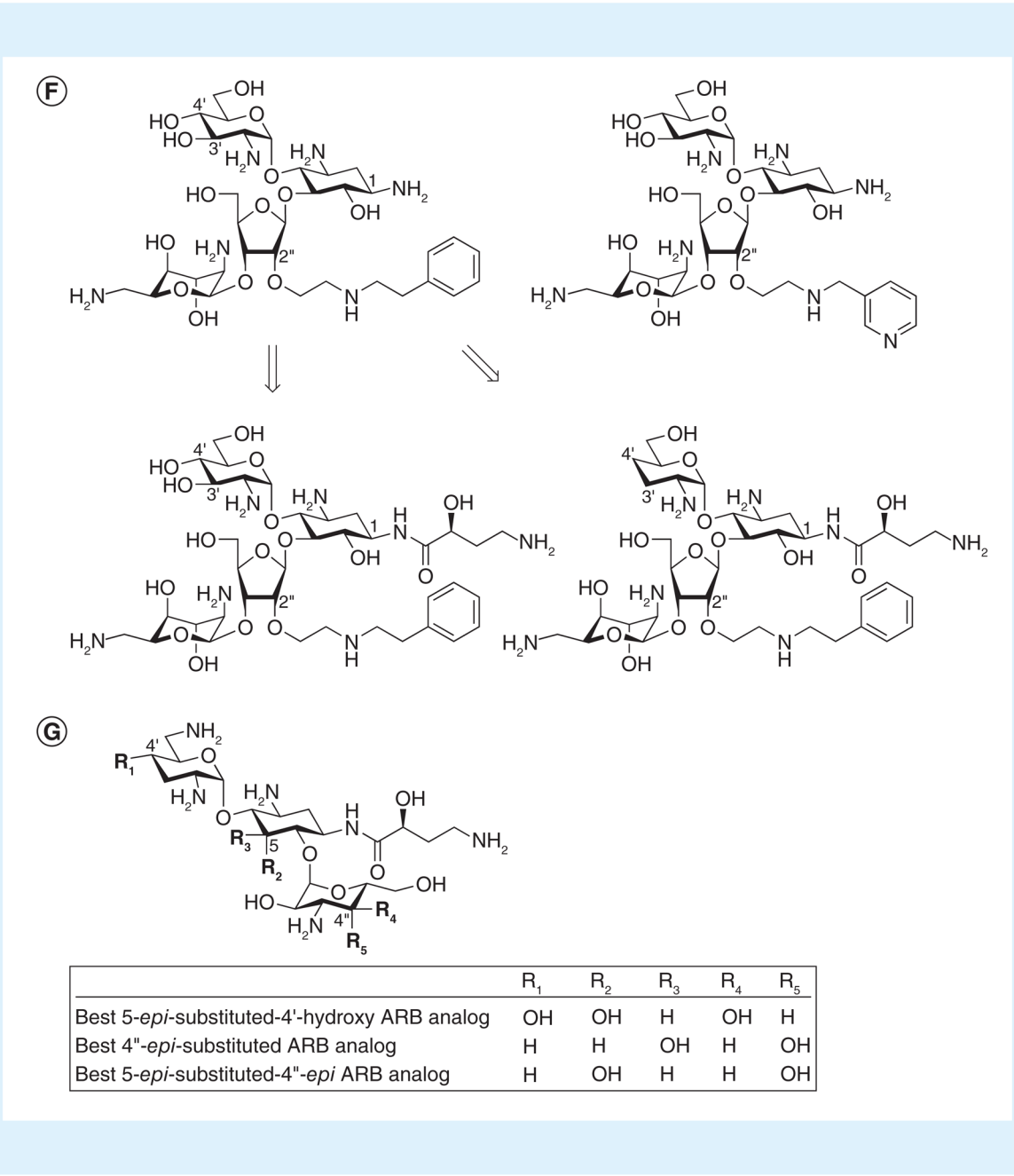


AG1-AG2	RNA binding	Antibacterial activity	AME substrate activity
NEA-NEA	√	√	√
NEB-NEB	√	√	X
NEO-NEO	√	X	X
NEO-TOB	√	X	X
TOB-TOB	√	X	X
KAN-KAN	√	X	X
KAN-TOB	√	X	X

Figure 6. Aminoglycoside dimers and conformationally constrained aminoglycoside analogs

(A) NEA–NEA dimers discussed. **(B)** Summary of aminoglycoside dimers synthesized and studied. **(C)** Hybrids of NEO or paromomycin with sisomicin. **(D)** Rigid NEO analogs with a 2′-5″ connections. **(E)** Rigid NEO analogs with a 6-OH to 6‴-NH tethers.
KAN: Kanamycin; NEA: Neamine; NEB: Nebramine; NEO: Neomycin B; TOB: Tobramycin.





	R_1	R_2	R_3	R_4	R_5
Best 5-*epi*-substituted-4'-hydroxy ARB analog	OH	OH	H	OH	H
Best 4"-*epi*-substituted ARB analog	H	H	OH	H	OH
Best 5-*epi*-substituted-4"-*epi* ARB analog	H	OH	H	H	OH

Figure 7. Aminoglycoside derivatives that evade the action of aminoglycoside-modifying enzymes
(A) Neamine derivatives modified by addition of an 4-amino-2-hydroxybutyryl group at position 1 and aliphatic amines at position 6. **(B)** 6,3′,4′-tri(2-naphthylmethylene)-neamine. **(C)** 4′-modified kanamycin B derivatives. **(D)** 6′-*N*-glycinyl-tobramycin. **(E)** 6″-thioether tobramycin analogs. **(F)** 2″-*O*-substituted paromomycin analogs. **(G)** 5-*epi*-substituted-4′-hydroxy-, 4″-*epi*- and 5-*epi*-derivatives of ARB.
ARB: Arbekacin.

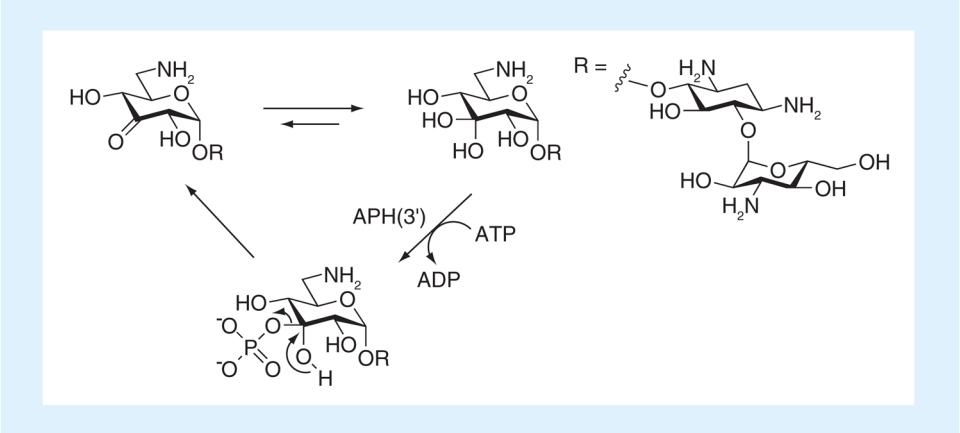


Figure 8.
'Self-regenerating' kanamycin A analogs that evade modification by aminoglycoside phosphotransferase (3′) enzymes.

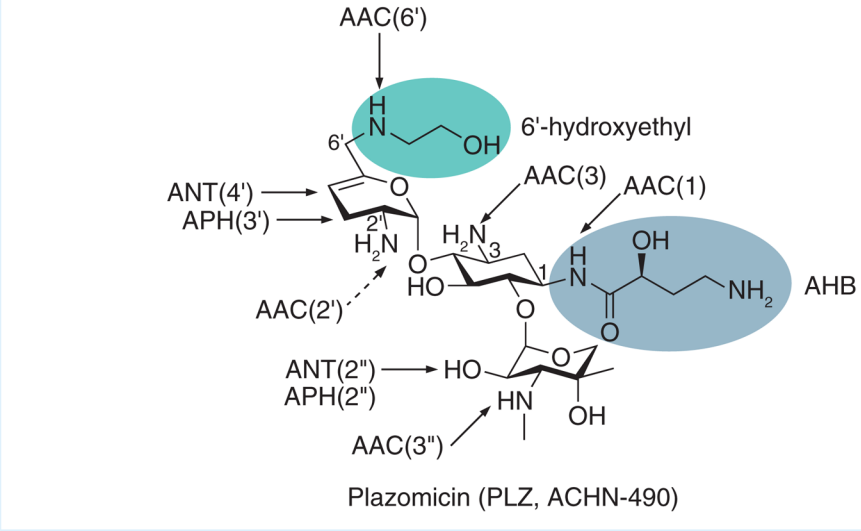


Figure 9. Plazomicin with potential sites of modifications by aminoglycoside-modifying enzymes indicated by arrows

The solid line arrows represent positions shown to be resistant to aminoglycoside-modifying enzyme modifications. The dotted arrow indicates the position still susceptible to modification by the aminoglycoside-modifying enzyme AAC(2′). Highlighted are the substitutions that differentiate plazomicin from sisomicin: the AHB at position 1 and the hydroxyethyl at position 6′.

AAC: Aminoglycoside acetyltransferase; AHB: 4-amino-2-hydroxybutyryl; ANT: Aminoglycoside nucleotidyltransferase; APH: Aminoglycoside phosphotransferase.

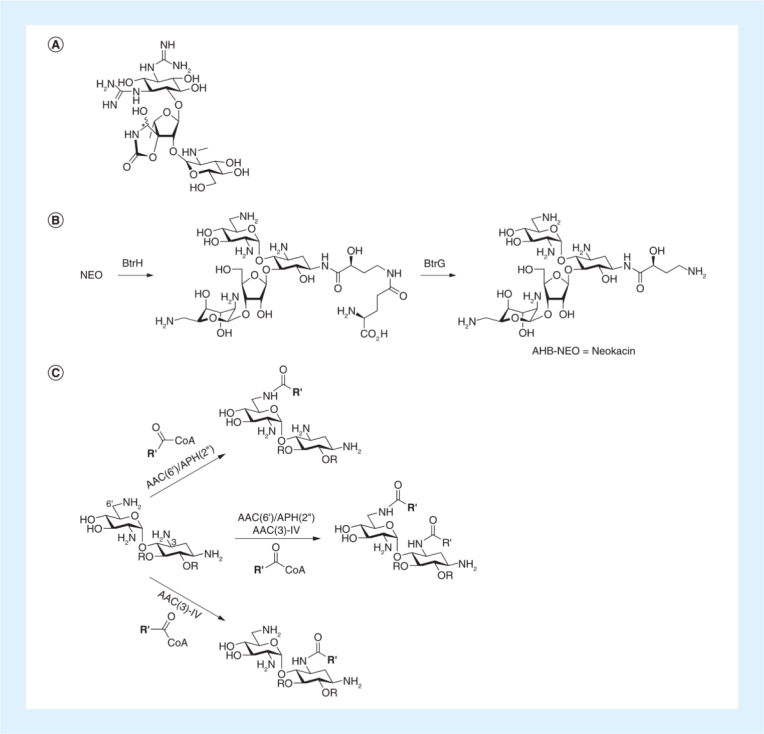


Figure 10. Biosynthesis and chemoenzymatic synthesis of novel aminoglycosides

(A) Biosynthetically produced rhodostreptomycin A and B. **(B)** Chemoenzymatic installation of an AHB moiety on NEO to produce neokacin. The conversion of kanamycin A to amikacin was similarly achieved using BtrH and BtrG. **(C)** Chemoenzymatic *N*-acylation of aminoglycosides by AACs. AAC: Aminoglycoside acetyltransferase; AHB: 4-amino-2-hydroxybutyryl; APH: Aminoglycoside phosphotransferase; NEO: Neomycin B.

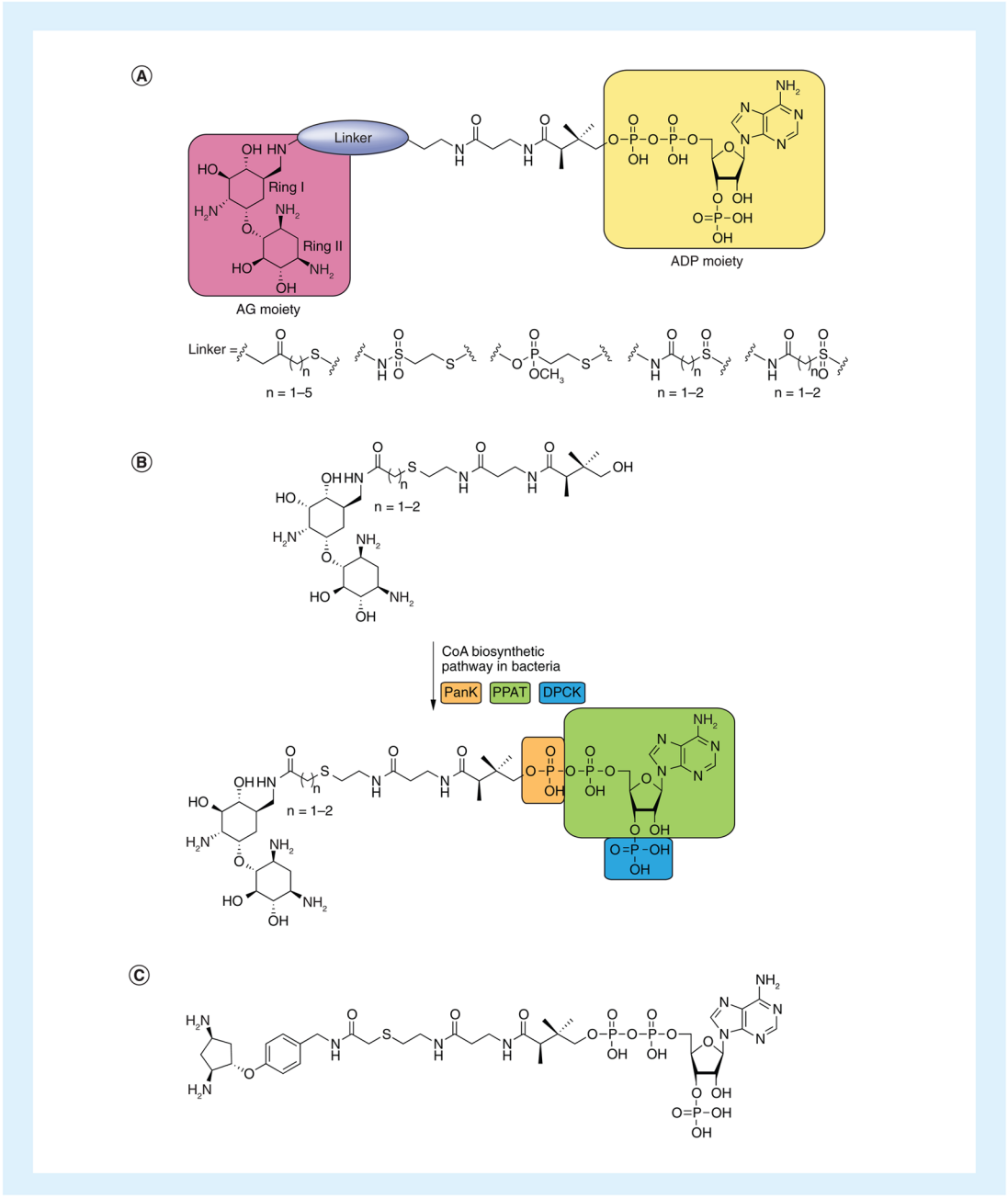


Figure 11. Aminoglycoside-AcCoA bisubstrates as aminoglycoside-modifying enzyme inhibitors

(A) A representative bisubstrate scaffold explored as an AG acetyltransferase (6′) inhibitor. Representative examples of linkers investigated are shown. **(B)** Bisubstrate prodrugs with increased bacterial membrane penetration generated biosynthetically *in vivo*. **(C)** Bisubstrate-inspired AG acetyltransferase (6′) inhibitor designed from NMR-guided fragment screens.

AG: Aminoglycoside.

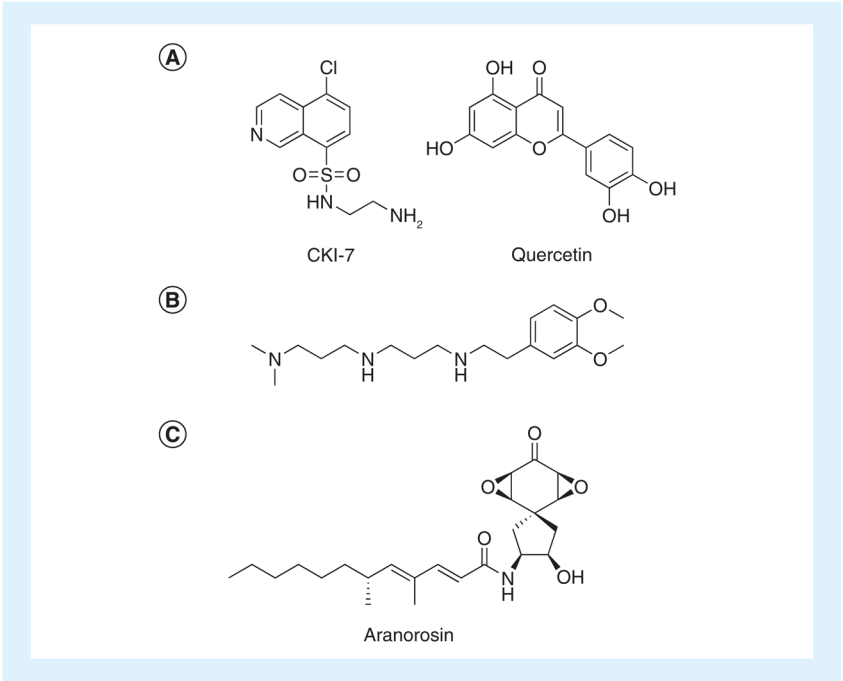


Figure 12. Aminoglycoside-modifying enzyme inhibitors

(A) Eukaryotic kinase inhibitors identified to also be APH inhibitors. **(B)** Non-carbohydrate aminoglycoside-modifying enzyme inhibitor containing a 1,3-diamine moiety to mimic that found in the scaffold of many aminoglycosides. **(C)** Aranorosin, a natural product reported to circumvent arbekacin resistance by inhibiting aminoglycoside acetyltransferase (6′)-Ie/aminoglycoside phosphotransferase (2″)-Ia.

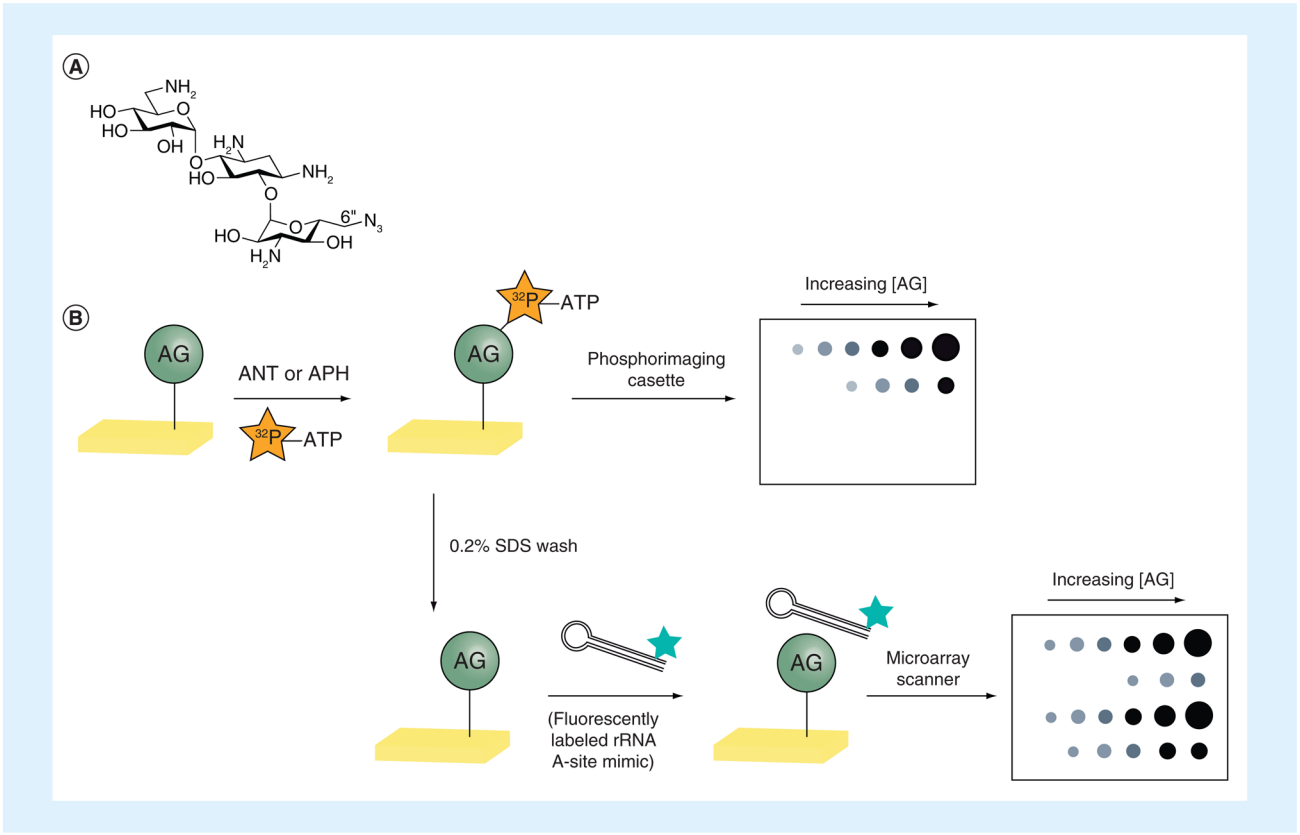


Figure 13. High-throughput microarrays

(A) 6″-azido–kanamycin A, an example of an aminoglycoside derivative with a chemical handle for Huisgen 1,3-dipolar cycloaddition attachment to alkyne-functionalized slides. **(B)** Microarray assay.

AG: Aminoglycoside; ANT: Aminoglycoside nucleotidyltransferase; APH: Aminoglycoside phosphotransferase.

Table 1

Summary of aminoglycoside acetyltransferases for which structures have been determined.

Position modified	Enzyme	PDB code	Substrate	Co-substrate	Bacterial species	Oligomeric state	Ref.
3	AAC(3)-Ia	1BO4	-	CoA	*Serratia marcescens*	Dimer	[143]
3	Putative AAC(3)	3E4F	-	-	*Bacillus anthracis*	Dimer	[144]
3	Putative AAC(3)	3IJW	-	CoA	*B. anthracis*	Dimer	[144]
3	Putative AAC(3)	3KZL	-	AcCoA	*B. anthracis*	Dimer	[144]
3	Putative AAC(3), H183G mutant	3N0M	-	AcCoA	*B. anthracis*	Dimer	[144]
3	Putative AAC(3), H183A mutant	3N0S	-	AcCoA	*B. anthracis*	Dimer	[144]
3	Putative AAC(3)	3SLB	Cytosine	AcCoA	*B. anthracis*	Dimer	
3	Putative AAC(3)	3SLF	Uracil	AcCoA	*B. anthracis*	Dimer	
2′	AAC(2′)-Ic	1M44			*Mycobacterium tuberculosis*	Dimer	[145]
2′	AAC(2′)-Ic	1M4D	TOB	CoA	*M. tuberculosis*	Dimer	[145]
2′	AAC(2′)-Ic	1M4G	RIB	CoA	*M. tuberculosis*	Dimer	[145]
2′	AAC(2′)-Ic	1M4I	KAN A	CoA	*M. tuberculosis*	Dimer	[145]
6′	AAC(6′)	2PBE	-	-	*Bacillus subtilis*	Dimer	
6′	AAC(6′)	3F5B	-	-	*Ligonella pneumophila*		
6′	AAC(6′)-Ib	1V0C	KAN C	AcCoA	*Escherichia coli*	Monomer	[146]
6′	AAC(6′)-Ib	2BUE	RIB	CoA	*E. coli*	Monomer	[146]
6′	AAC(6′)-Ib	2VQY	PAR	AcCoA	*E. coli*	Monomer	[146]
6′	AAC(6′)-Ig	4EVY	TOB	-	*Acinetobacer haemolyticus*	Dimer	
6′	AAC(6′)-Ig	4F0Y	-	-	*A. haemolyticus*	Dimer	
6′	AAC(6′)-Ih	4E8O	-	-	*Acinetobacter baumannii*	Dimer	
6′	AAC(6′)-Ii	1B87	-	AcCoA	*Enterococcus faecium*	Dimer	[147]
6′	AAC(6′)-Ii	1N71	-	CoA	*E. faecium*	Dimer	[148]
6′	AAC(6′)-Ii	2A4N	-	CoA	*E. faecium*	Dimer	[149]
6′	AAC(6′)-Iy	1S3Z	RIB	CoA	*Salmonella enteritidis*	Dimer	[150]
6′	AAC(6′)-Iy	1S5K	-	CoA	*S. enteritidis*	Dimer	[150]
6′	AAC(6′)-Iy	1S60	-	CoA	*S. enteritidis*	Dimer	[150]
6′	AAC(6′)-Iy	2VBQ	-	CoA analog	*S. enterica*	Dimer	[102]

Position modified	Enzyme	PDB code	Substrate	Co-substrate	Bacterial species	Oligomeric state	Ref.
1, 2′, 6′ of NEA	Eis	3R1K	-	CoA	*M. tuberculosis*	Hexamer	[22]
ND		3RYO	-	AcCoA	*M. tuberculosis*	Hexamer	[24]
ND		3UY5	-	-	*M. tuberculosis*	Hexamer	[24]
ND		3SXN	-	CoA	*M. smegmatis*	Hexamer	[24]
ND		2OZG	-	CoA	*Anabaena variabilis*	Hexamer	[26]
ND		3N7Z			*B. anthracis*	Hexamer	

AAC: Aminoglycoside acetyltransferase; KAN: Kanamycin; ND: Not determined; NEA: Neamine; PAR: Paromomycin; RIB: Ribostamycin; TOB: Tobramycin.

Table 2

Summary of aminoglycoside phosphotransferases for which structures have been determined.

Position modified	Enzyme	PDB code	Substrate	Cosubstrate	Bacterial species	Oligomeric state	Ref.
3′	APH(3′)-IIa	1ND4	KAN A	-	*Klebsiella pneumoniae*	Dimer	[151]
3′	APH(3′)-Ia	4EJ7	-	ATP	*Acinetobacter baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4FEU	KAN A + inhibitor SP600125	-	*A. baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4FEV	KAN A + inhibitor PP1	-	*A. baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4FEW	KAN A + inhibitor PP2	-	*A. baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4FEX	KAN A + inhibitor AG1478	-	*A. baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4GKH	KAN A + inhibitor 1-NA-PP1	-	*A. baumannii AYE*	Dimer	
3′	APH(3′)-Ia	4GKI	KAN A + inhibitor 1-NM-PP1	-	*A. baumannii AYE*	Dimer	
3′/5″	APH(3′)-IIIa	2BKK	Ankyrin repeat inhibitor protein	ADP	*Enterococcus faecalis*	Monomer	[109]
3′/5″	APH(3′)-IIIa	3Q2J	CKI-7 (protein kinase inhibitor)	ADP	*E. faecalis*	Monomer	[111]
3′/5″	APH(3′)-IIIa	1J7I	-	-	*E. faecalis*	Dimer	[152]
3′/5″	APH(3′)-IIIa	1J7L	-	ADP	*E. faecalis*	Dimer	[152]
3′/5″	APH(3′)-IIIa	1J7U	-	AMPPNP	*E. faecalis*	Dimer	[152]
3′/5″	APH(3′)-IIIa	1L8T	KAN A	ADP	*E. faecalis*	Dimer	[153]
3′/5″	APH(3′)-IIIa	2B0Q	NEO	ADP	*E. faecalis*	Dimer	[153]
3′/5″	APH(3′)-IIIa	3TM0	BUT A	AMPPNP	*E. faecalis*	Monomer	[154]
2″	APH(2″)-Ib	4DCA	-	ADP	*Escherichia coli*	Monomer	
2″	APH(2″)-IIa	3HAM	GEN	-	*Enterococcus faecium*	Dimer	[155]
2″	APH(2″)-IIa	3HAV	STR	ATP	*E. faecium*	Monomer	[155]
2″	APH(2″)-IIIa	3TDV	-	GDP	*Enterococcus gallinarum*	Dimer	[29]
2″	APH(2″)-IIIa F108L mutant	3TDW	-	GDP	*E. gallinarum*	Dimer	[29]
2″	APH(2″)-IVa form I	3N4T	-	-	*Enterococcus casseliflavus*	Monomer	[156]
2″	APH(2″)-IVa form II	3N4U	-	-	*E. casseliflavus*	Monomer	[156]
2″	APH(2″)-IVa form III	3N4V	-	-	*E. casseliflavus*	Monomer	[156]
2″	APH(2″)-IVa	3SG8	TOB	-	*E. casseliflavus*	Dimer	[157]
2″	APH(2″)-IVa	3SG9	KAN A	-	*E. casseliflavus*	Dimer	[157]
2″	APH(2″)-IVa	3SGC	-	-	*E. casseliflavus*	Dimer	[157]

Position modified	Enzyme	PDB code	Substrate	Cosubstrate	Bacterial species	Oligomeric state	Ref.
2″	APH(2″)-Ib	3UZR	-	-	*E. coli*	Monomer	
2″	APH(2″)-Id/ APH(2″)-IVa	4DBX	-	-	*E. casseliflavus*	Monomer	[112]
2″	APH(2″)-Id/ APH(2″)-IVa	4DE4	-†	-	*E. casseliflavus*	Dimer	[112]
2″	APH(2″)-Id/ APH(2″)-IVa	4DFB	KAN A	-	*E. casseliflavus*	Dimer	[112]
2″	APH(2″)-Id/ APH(2″)-IVa	4DFU	KAN A + quercetin inhibitor	-	*E. casseliflavus*	Dimer	[112]
2″	APH(2″)-IVa	4DT8	-	Adenosine	*E. casseliflavus*	Dimer	[27]
2″	APH(2″)-IVa	4DT9	-	Guanosine	*E. casseliflavus*	Dimer	[27]
2″	APH(2″)-IVa F95M mutant	4DTA	-	Adenosine	*E. casseliflavus*	Dimer	[27]
2″	APH(2″)-IVa F95Y mutant	4DTB	-	Guanosine	*E. casseliflavus*	Dimer	[27]
4	APH(4)-Ia	3TYK	HYG B variant	-	*E. coli*	Monomer	[158]
9	APH(9)-Ia	3I0O	SPT	ADP	*Ligonella pneumophilia*	Dimer	[159]
9	APH(9)-Ia	3I0Q	-	AMP	*L. pneumophilia*	Dimer	[159]
9	APH(9)-Ia	3I1A	-	-	*L. pneumophilia*	Dimer	[159]
9	APH(9)-Ia	3Q2M	CKI-7 (protein kinase inhibitor)	-	*L. pneumophila* 130b	Dimer	[111]
Putative	APH	3CSV	-	-	*Silicibacter* sp. TM1040	Dimer	
Putative	APH	3DXP	-	-	*Ralstonia eutropha* JMP134	Dimer	

†Complex with HEPES.

AMPPNP: Adenosine 5′-(β,γ-imido)triphosphate; APH: Aminoglycoside phosphotransferase; BUT: Butirosin; GEN: Gentamicin; KAN: Kanamycin; NEO: Neomycin B; SPT: Spectinomycin; STR: Streptomycin; TOB: Tobramycin.

Table 3

Summary of aminoglycoside nucleotidyltransferases for which structures have been determined.

Position modified	Enzyme	PDB code	Substrate	Cosubstrate	Bacterial species	Oligomeric state	Ref.
4′	ANT(4′)	1KNY	KAN A	AMPPCP	*Staphylococcus aureus*	Dimer	[160]
4′	ANT(4′)-IIb	4EBJ	-	-	*Pseudomonas aeruginosa*	Dimer	
4′	ANT(4′)-IIb	4EBK	TOB	-	*P. aeruginosa*	Dimer	

AMPPCP: β,γ-methyleneadenosine 5′-triphosphate; ANT: Aminoglycoside nucleotidyltransferase; KAN: Kanamycin; TOB: Tobramycin.