Understanding and overcoming aminoglycoside resistance caused by N-6'-acetyltransferase

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Abstract

Aminoglycosides occupy a special niche amongst antibiotics in part because of their broad spectrum of action. Bacterial resistance is however menacing to render these drugs obsolete. A significant amount of work has been devoted to understand and overcome aminoglycoside resistance. This mini-review will discuss aminoglycoside-modifying enzymes (AMEs), with a special emphasis on the efforts to comprehend and block resistance caused by aminoglycoside 6'-N-acetyltransferase (AAC(6')).

Graphical Abstract

Resistance-causing AAC(6')s have been studied and avoided in a number of ways. AAC(6')-Ii surprises everyone with its complex allosteric behaviour.

1. INTRODUCTION

1.1 Antibiotics and human health

Bacteria can be beneficial or harmful to human health. On one hand, bacteria make up the majority of the human microbiome, where they are involved in a mutualistic symbiotic relationship that provides nutritional, protective, metabolic and digestive benefits to the host.

¹ On the other hand, bacteria can be pathogenic to humans, causing diseases that claim an unaccountable number of human lives. In 1900 only, an estimated 19.4 million deaths in Western Europe were attributed to tuberculosis, a deadly infection caused by *Mycobacterium tuberculosis*.²

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Following the discovery and clinical usage of antibiotics, human health has improved significantly. Antibiotics are compounds that have selective toxicity towards bacteria, and may either kill them (bactericidal effect) or stunt their growth (bacteriostatic). Streptomycin, an aminoglycoside discovered in 1943, was the first effective remedy for the treatment of tuberculosis and was the reason for the rapid decline of deaths caused by this disease worldwide.³ The problem, however, is that bacteria evolve rapidly and eventually develop resistance to antibiotics. Through evolution, bacteria have developed various resistance mechanisms, such as target modification, molecular bypass, efflux, and enzyme-catalyzed chemical modification.^{4–6} Augmenting this problem is the natural ability of bacteria to acquire resistance-causing genes through horizontal transfer between bacterial strains and species. Overall, this has led to the rapid emergence of multi-resistant strains of bacteria that are becoming an urgent medical health issue. For example, studies show that in 2005 nearly 19,000 people died in the United States by causes related to drug-resistant bacterial infections.⁷ This figure translates into more deaths per year from resistant infections in the US than fatalities attributed to either of HIV/AIDS, Parkinson's disease, emphysema, or homicide.⁸

1.2 Aminoglycosides and bacterial resistance

Aminoglycosides are an important class of drugs used in the treatment of serious infections caused by aerobic Gram-negative and Gram-positive bacteria. Their mode of action relies mainly on targeting the major groove of the 16S rRNA subunit of the bacterial ribosome. Binding of the aminoglycoside causes misreading of the genetic code, producing misfolded proteins, and eventual cell death.^{9–11} Despite being among the oldest antibacterial agents to date, aminoglycosides have maintained significant effectiveness throughout the ages and remain commonly used antibiotics.¹² Bacterial resistance to aminoglycosides, however, is spreading rapidly. The earliest accounts of resistance can be dated back to 1946, when doctors at the Mayo Clinic observed patients with tuberculosis carrying strains that were a thousand times more resistant to streptomycin.^{13, 14}

Aminoglycosides are natural products from bacteria of the *Streptomyces* or *Micromonospora* genera.¹⁵ Aminoglycoside producers typically avoid committing suicide by methylating their 16S rRNA, which prevents key interactions with the antibacterial agent.¹⁶ In the clinics, however, this mechanism of resistance is not widespread, although it is on the rise.^{17, 18} Currently, the most prominent mechanism of resistance to aminoglycosides entails bacterial expression of aminoglycoside-modifying enzymes (AMEs). The three classes of enzymes that confer aminoglycoside resistance are adenylyltransferases (ANTs), *O*-phosphoryltransferases (APHs), and *N*-acetyltransferases (AACs). Numerous excellent reviews covering aminoglycosides and aminoglycoside-modifying enzymes have recently been published.^{19–27}

Among the AMEs, the class of AAC enzymes are widespread in the clinics.^{28, 29} AAC(6'), for example, is known to catalyze the addition of an acetyl group from AcCoA to the 6'-*N* of aminoglycosides (Figure 1). This modification disrupts the crucial electrostatic and hydrogen bonding interactions between the 6'-NH₂ of the aminoglycoside and A1408 of the 16S rRNA (Figure 1).³⁰ The kinetic mechanism of AACs follow either an ordered sequential

mechanism AAC(6′)-Ib,³¹ AAC(3)-Ib/AAC(6′)-Ib′,³² and ANT(3′′)-Ii/AAC(6′)-IId³³, or a random sequential mechanism AAC(6′)-Iy,³⁴ AAC(2′)-Ic,³⁵ APH(2′′)-AAC(6′),³⁶ AAC(3)-IV,³⁷ and AAC(3)-I³⁸

Several crystal structures are available for AACs. These include structures of AAC(6′)-Iy from *Salmonella enterica* in complex with CoA,³⁹ or CoA and ribostamycin,³⁹ *Klebsiella pneumoniae* AAC(6′)-Ib in complex with CoA,⁴⁰ ribostamycin,³¹ or kanamycin,⁴⁰ or AcCoA and either paromomycin or kanamycin C.³¹ The broad spectrum variant, AAC(6′)-Ib₁₁⁴¹ was also crystallized without any substrate,⁴⁰ as were the AAC(6′) isoform from *Legionella pneumophila* (pdb 3F5B), and a multi-acetylating acetyltransferase from *M. tuberculosis*, Eis.⁴² There are structures reported for AAC(2′)-Ic from *M. tuberculosis*, in the apo form, and in complex with CoA and either ribostamycin, tobramycin or kanamycin A,⁴³ as well as AAC(3)-Ia from *Serratia marcescens* in complex with CoA⁴⁴, and the AAC(3) isoform from *Bacillus subtilis* with CoA (pdb 2NYG). Crystal structures of *E. faecium* AAC(6′)-Ii in complex with AcCoA⁴⁵ or CoA^{46, 47} are also reported.

This mini review mainly focuses on mechanistic studies of AAC(6′)s, and strategies exploited to counteract or inhibit the effects of these resistance-causing enzymes.

2. MECHANISTIC STUDIES OF AAC(6′)s

A better mechanistic understanding of AAC(6′)s is desirable if one hopes to overcome the effect of these enzymes. Some of the more studied AAC(6′) isoforms are AAC(6′)-Ib and AAC(6′)-Ii. AAC(6′)-Ib is a 200 amino acid protein (24.5 kDa monomer) that is plasmid-encoded and was first identified in *Klebsiella pneumonia* isolates,^{48, 49} but is also harbored by several Gram-negative strains of *Acinetobacter*, *Pseudomonadaceae*, *Enterobacteriaceae*, and *Vibrionaceae*.⁵⁰ It is estimated that about 70% of AAC(6′)-producing Gram-negative clinical isolates carry the AAC(6′)-Ib gene.⁵¹ AAC(6′)-Ib is typically monomeric,⁴⁰ and uses Asp115 to keep the 6′-NH₂ of aminoglycosides deprotonated.³¹ A number of AAC(6′)-Ib variants have also been reported. For example, AAC(6′)-Ib-cr has drawn a lot of attention because of its ability to acetylate, and thus deactivate, fluoroquinolones, in addition to amikacin and other aminoglycosides.³¹ It is suggested that the two mutations, Trp102Arg and Asp179Tyr, are key to enabling fluoroquinolones to bind into the active site of AAC(6′)-Ib-cr. Another variant, AAC(6′)-Ib_{II}, is characterized by a broader spectrum of resistance,⁴¹ this time attributed to Leu118 and Ser119.

In general however, AAC(6′)-Ib has shown low tolerance to amino acid substitutions. Studies to identify the catalytic residues have revealed that mutation of E167,⁵² D117,⁵³ L120,⁵³ or F171⁵⁴ typically leads to complete loss of resistance activity. Several other mutations of AAC(6′)-Ib also affects activity to various extent.^{52, 55} An interesting mutagenic study reported aimed at delineating the differences in aminoglycoside substrate specificity between AAC(6′)-Ib and AAC(6′)-IIa.⁵⁶ The class-I AAC(6′) enzymes acetylate amikacin and but not gentamicin C₁. On the other hand, the class-II enzymes are capable of acetylating gentamicins C₁, C_{1a} and C₂, but not amikacin.⁵¹ Mutation of Leu119 to Ser in AAC(6′)-Ib partially reversed its resistance profile, with the activity towards gentamicin C₁ increasing, at the cost of lowered amikacin activity. On the other hand, the Ser119Leu

mutation in AAC(6')-IIa resulted in raised amikacin activity but with a lower gentamicin C₁ activity.

AAC(6')-II is a 182 amino acid protein (41.4 kDa homodimer) that is chromosomally encoded in *Enterococcus faecium*, one of the leading causes of hospital-acquired infections.⁵⁷ Catalysis by AAC(6')-II was suggested to proceed via an ordered bi-bi mechanism, where AcCoA must bind the enzyme before the aminoglycoside.⁵⁸ A monomeric variant has been generated with the single mutation Trp104Ala.⁵⁸ Isothermal titration calorimetry studies revealed two non-equivalent aminoglycoside binding sites for the AAC(6')-II dimer, but only one binding site for the Trp104Ala mutant. Another study identified Glu72 and Leu76 as important residues.⁵⁹ Mutation of Glu72, caused a dramatic reduction of affinity for both 4,5- and 4,6-disubstituted aminoglycosides, yet no effect on the binding of the poly-L-lysine substrate. Leu76 on the other hand, seems to influence both the interaction of the enzyme with acetyl-CoA and stabilization of the transition state. While the Leu76Ala mutation does not affect catalysis, the Leu76Pro mutant is catalytically inactive. This was rationalized based on the lack of hydrogen bonding capacity of the backbone NH group (with Pro), which may impinge on transition state stabilization.

The high promiscuity of AAC(6')s towards various aminoglycosides is intriguing. Work by Serpersu and coworkers has provided some insight on the conformation adopted by aminoglycosides when in complex with AAC(6')-II.⁶⁰ NMR spectroscopy and molecular modeling studies revealed two major enzyme-bound conformers for isepamicin and one for butirosin A. When these conformers were superimposed at the 2-deoxystreptamine ring, one of the isepamicin conformers was deemed non-conducive to acetylation. This result offers one explanation for isepamicin remaining one of the poorest AME substrates.⁶¹

Due to its complex allosteric behaviour, AAC(6')-II has turned out to be an excellent model for studies of protein allostery. On one hand, the ¹⁵N/¹H NMR correlation spectrum of this enzyme in the absence of ligands shows broadened and poorly resolved peaks, which is characteristic of a highly dynamic or partially unfolded state. The addition of saturating amounts of AcCoA⁶² results in much sharper peaks and well-resolved spectra, indicating a shift to a well-defined 3D structure. Unexpectedly, binding of an aminoglycoside (instead of AcCoA) to AAC(6')-II results in folding of the enzyme into a significantly different conformation.⁶³ In order to further study the allosteric effects observed upon binding of the substrate AcCoA to this enzyme, a new approach combining NMR, ITC and CD was reported.⁶⁴ This work was not possible without a novel ITC method using variable-*c*,^{62, 65} as well as a general method for global analyses of variable temperature ITC data.⁶⁶ Titration experiments (NMR and ITC) combined with melting curves (CD) demonstrate that homotropic allostery between the two active sites of the homodimeric enzyme is modulated by opposing mechanisms. On one hand negative cooperativity is driven by a mechanism akin to the Hilser and Thompson model (HT),⁶⁷ whereas positive cooperativity follows the classical Koshland-Nemethy-Filmer (KNF) model.⁶⁸ In the KNF model, free and bound subunits adopt different conformations, and cooperativity results from inter-subunit interactions (Figure 2A). Hilser and Thompson (HT) have proposed a model where the subunits of the enzyme exist in conformational equilibria between unfolded and folded states. Upon ligand binding to a folded subunit, induction of conformational changes at the

other subunit is promoted in folding-dependent positive or negative cooperativity (Figure 2B). When AAC(6')-Ii binds AcCoA, one subunit folds into a bound conformation which is reminiscent to a KNF folding model. This destabilizes the interactions between the two subunits and reduces the unbound subunits stability, causing it to unfold at lower temperatures. This produces opposing cooperative events which cause AcCoA to bind with positive cooperativity at lower temperatures and negative cooperativity at higher temperatures (Figure 2C). Interestingly, preliminary studies of the interaction of the enzyme with its other substrate, the aminoglycoside, suggest that the enzyme's behaviour is equally complex yet fairly different.⁶³ In this case, the data points to binding events reminiscent of the Monod-Wyman-Changeux (MWC) binding model.⁶⁹ At this point it is still unclear if such a complex allosteric behavior is widespread amongst AMEs, or even other enzymes. Undoubtedly, however, a better understanding of enzyme mechanisms will assist efforts to surmount resistance.

3. AMINOGLYCOSIDE ANALOGUES DESIGNED TO OVERCOME AAC(6')s

Numerous studies have aimed at synthesizing aminoglycoside analogues that are not transformed by resistance-causing enzymes, yet retain the ability to bind to the bacterial 16S rRNA.^{70–72} This area of research is too broad to be covered here but an overview is provided for aminoglycoside derivatives that have been tested against AAC(6') and reported not to be substrates of the enzyme.

3.1. Aminoglycoside N-6' derivatives

One of the earliest reports of aminoglycoside derivatives active against AAC(6')-expressing bacterial strains was available more than 30 years ago.^{73, 74} Kanamycin B derivatives **1a-c** and the 3',4'-dideoxykanamycin B derivative **1d** (Figure 3A) are active against *Escherichia coli* K-12 R5 and *Pseudomonas aeruginosa* GN315, both of which express AAC(6') enzymes. Aminoglycosides normally bind the bacterial 16S rRNA with one of the key binding interactions between the 6'-NH₂ of the aminoglycoside and the N-1 of A1408 (Figure 1), as revealed by crystal structures of aminoglycoside-RNA complexes.^{75–77} Following acetylation by AAC(6')s, this key interaction with A1408 is disrupted. Besides the alkyl groups of **1a-d**, several other functionalities have been introduced at the 6'-NH₂ in order to prevent or slow acetylation, however many were either too bulky or lacked functionalities required for hydrogen bonding with A1408.^{70, 78–83}

To address this issue, compounds **2a-b** (Figure 3B) were designed to display an N-6' substituent containing a hydrogen bond donor for interaction with A1408, and attachment of this group via an amide bond was expected to prevent acetylation by AAC(6').⁸⁴ As expected, compounds **2a-b** were not substrates of AAC(6')-Ii. However, bacterial studies with *E. coli* revealed that antibacterial activity was also compromised to some extent compared to neamine.

An aminoglycoside microassay was developed to screen for aminoglycoside analogues that may potentially bind AAC(6')-Iy and AAC(2')-Ic with high affinity.⁸⁵ The library used consisted of guanidinoglycosides,⁸⁶ which were considered on the basis that 1) they are easily synthesized, and 2) the introduction of positively charged guanidino groups was

expected to promote stronger binding to the anionic aminoglycoside binding pocket of rRNA. From a list of commonly known aminoglycosides such as kanamycin A, neomycin, ribostamycin, paromomycin, and lividomycin, a series of guanidinoglycosides were synthesized. Following immobilization of the β -Ala-guanidinoglycosides to the microarray, incubation was carried out with fluorescently labelled AAC(6')-Iy and AAC(2')-Ic to determine binding. In all cases, stronger binding to AAC(6') was observed with the β -Ala-guanidinoglycosides (e.g. compound **3b**) compared to their corresponding β -Ala-aminoglycosides (e.g. compound **3a**), (Figure 3C). The most potent compound of this series, **3b**, was not a substrate for either AAC(6')-Iy or AAC(2')-Ic, whereas its corresponding aminoglycoside, ribostamycin, is completely consumed by both enzymes after 10 minutes. Observations also concluded that **3b** acts as a noncompetitive inhibitor of AAC(6')-Iy, with K_{ij} and K_{is} values in the range of 20–100 μ M.

A very promising aminoglycoside derivative modified at N -6' with a functionality that may interact with A1408 is compound **4**, or ACHN-490. Compound **4** is being developed by Achaogen under the name plazomicin (Figure 3D),^{70, 87, 88} and is currently in Phase II clinical trials for complicated urinary tract infections and acute pyelonephritis. Derived from sisomicin, **4** was shown to be active against several bacterial strains including a number of strains harbouring an AAC(6')-encoding gene.⁸⁷

3.2 Aminoglycoside dimers

Research has shown that neamine binds the biotinylated *E. coli* 16S rRNA A-site in a 2:1 complex with a K_d of 10 μ M for each binding site.⁸⁹ With this in mind, neamine dimers with various linkers and moieties were synthesized in hopes of 1) improving binding affinity to the A-site and, 2) escaping or inhibiting the action of AMEs. From the library of neamine dimers reported, compounds **5a**, **5b** and **6** (Figure 3E) were found to be the most promising, with K_d values against the A site of 1.1, 0.8, and 0.04 μ M, respectively, which correspond to improvements of 10-fold compared to “monomeric” neamine. When tested as substrates for AAC(6')-Ii, K_M values of 53.4, 83.6, and 28.7 μ M, were found for compounds **5a**, **5b** and **6**, respectively. The dimers are therefore poorer substrates than neamine (5.82 μ M).⁵⁷ When tested against *E. coli* for antibacterial activity, **5a** and **5b** displayed similar activity to that of neamine, and **6** was 8–16 fold more effective.

4. BLOCKING AAC(6')s

Inhibition of AMEs is a focus of many studies aiming to develop treatments against resistant strains of bacteria. Several inhibitors have been reported for ANT(2''),^{90–92} APH(2'')-Ia,⁹³ APH(9)-Ia,⁹³ AAC(3)-I,⁹⁴ the bifunctional AAC(6')-APH(2''),^{89, 95, 96} and Eis identified from *M. Tuberculosis*,⁹⁷ yet the majority of inhibitors reported target APH(3'). Among these are compounds belonging to the isoquinoline-sulfonamides family,^{93, 98, 99} the ATP analogue 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine,¹⁰⁰ cationic antimicrobial peptides,⁹⁶ non-carbohydrate inhibitors,⁹² neamine-adenosine bisubstrates,¹⁰¹ covalent inhibitors such as bromoacetylated neamine analogues,¹⁰² and 2'-deamino-2'-nitro neamine and kanamycin B derivatives that act as mechanism-based inhibitors.¹⁰³ Alternative approaches to inhibit

APH(3')s include the generation of aminoglycoside analogues capable of self-regeneration following APH(3') modification,¹⁰⁴ and an allosteric ankyrin repeat inhibitory protein.¹⁰⁵

The approaches exploited to block AAC(6')s include: (1) small molecule inhibitors that act to competitively inhibit AAC(6')s, and (2) external guide sequences that downregulate the expression of AAC(6') enzymes.

4.1 Small molecule inhibitors

One common approach to overcome AMEs is to directly inhibit their activity through small molecules, and thus resensitize bacteria to co-administered aminoglycosides. There has been much research done to develop small molecule inhibitors of AAC(6')s, including the isoforms AAC(6')-Iy¹⁰⁶, AAC(6')-Ii,^{107–112} and AAC(6')-Ib.^{113, 114}

4.1.1 Cationic inhibitors—Owing to the anionic environment of the aminoglycoside binding pocket in AMEs, Wright and co-workers reasoned that cationic peptides would be good lead compounds towards developing broad-spectrum inhibitors of AMEs.⁹⁶ An added advantage of cationic peptides is that many of them display significant antimicrobial activity.¹¹⁵ A series of peptides were tested against AAC(6')-Ii, AAC(6')-Ie-APH(2'') and APH(3')-IIIa and many were shown to have low micromolar inhibition against these resistance-causing enzymes (Table 1). However, identifying a broad-spectrum inhibitor was difficult as the inhibition profiles of the peptides against the various enzymes did not always follow a recognizable trend. For example, indolicidin, CP11CN, and CP10A were inhibitors of AAC(6')-Ii, but not AAC(6')-Ie. On the other hand, peptides CP29 and CP2600 had activity against AAC(6')-Ie, but not AAC(6')-Ii. An important observation from these results is that a larger positive charge on the peptide does not correlate to better inhibition. To further probe the interaction between the peptides and the enzymes, truncated CP10A derivatives were prepared.⁹⁶ Maximum inhibition required the full length CP10A peptide, regardless of the charge of the removed amino acid. It was also suggested that the positioning of the positive charge in these peptides has little impact on inhibitory activity. None of the cationic peptides were shown to potentiate the antimicrobial effects of an aminoglycoside against bacteria harboring a resistance gene.

4.1.2 Bisubstrate inhibitors for mechanistic studies—To circumvent the lack of structural information about the aminoglycoside binding site of AAC(6')-Ii, aminoglycoside-CoA bisubstrates **7a-e** were synthesized (Figure 4A) through a regioselective and chemoselective one-pot method that avoids the necessity of functional group protection.¹⁰⁷ These molecules were successfully crystallized with AAC(6')-Ii and provided the first 3D structure of the aminoglycoside binding pocket. One of these bisubstrates, compound **7a**, has also been crystallized with the *S. enterica* isoform, AAC(6')-Iy.¹⁰⁶

Besides providing valuable insight about the binding orientation of the substrates and catalysis, bisubstrates **7a-e** were found to be potent inhibitors of aminoglycoside acetyltransferases (Figure 4A). Compounds **7a-c** are nanomolar inhibitors of AAC(6')-Ii, while compounds **7d-e** are micromolar inhibitors. In general, the trend observed suggests that shorter linkers yield better AAC(6')-Ii inhibitors. Compounds **7a-c** were also tested

against the AAC(6′)-I_y isoform, and showed low micromolar inhibition.¹⁰⁶ Studies with bifunctional AAC-6′-APH-2″ showed micromolar inhibition by some bisubstrates, and no transformation by the enzyme (unpublished data).

4.1.3 Modifications to the linker functionality in bisubstrate inhibitors—For the bisubstrates **7a-e**, the linkers between the aminoglycoside and CoA are restricted to short aliphatic amides. As for most AACs, data suggest that catalysis by AAC(6′)-I_i proceeds via nucleophilic attack of the 6′-NH₂ group of the aminoglycoside onto the thioester carbonyl of AcCoA to form a tetrahedral intermediate (Figure 5). The amide bond of bisubstrates **7a-e** is however not tetrahedral and one could postulate that bisubstrates with a linker containing an atom of tetrahedral geometry should be a better inhibitor. Moreover, such inhibitors represent mechanistic probes to validate the theory that AAC(6′) enzymes promote catalysis by stabilization of the tetrahedral intermediate.

Sulfonamides display higher polarizability at the S=O bond compared to the amide C=O bond, and a tetrahedral geometry at the sulfur atom. As a result, sulfonamides can be good mimics of tetrahedral intermediates. Sulfonamide-linked bisubstrates such as **2** were synthesized with the hope that they would show superior AAC(6′) inhibition activity (Figure 4B).¹⁰⁹ Inhibition studies, however, revealed that in comparison to bisubstrate **7b**, compound **8** is less potent.

Phosphonates have been more extensively used to mimic tetrahedral intermediates^{116–123} than sulfonamides.^{124–127} Bisubstrate **9** containing a phosphonate linker was prepared for comparison (Figure 4B). Poor solubility of CoA in organic solvents, combined with the incompatibility of P^{III} chemistry in aqueous conditions, as well as the presence of multiple functional groups on the substrates, posed a challenge for the preparation of **9**. These problems were circumvented via a Michael-type addition onto a vinyl-phosphonate in water.¹¹⁰ As with sulfonamides, the introduction of a tetrahedral phosphonate linker did not lead to improved inhibition, but rather to a 50-fold loss of potency compared to bisubstrate **7b**. The failure of compounds **8** and **9** to show improved inhibition activity against AAC(6′) suggests two possible conclusions. Either the sulfonamide- and phosphonate-containing compounds are poor mimics of the tetrahedral intermediate (maybe due to linker length for example), or the enzyme does not stabilize the proposed tetrahedral intermediate and may in fact act solely through proximity catalysis, as suggested by earlier reports.^{58, 59}

Sulfur oxidation was also explored to generate bisubstrates with yet another group of linkers. This was motivated by analysis of the crystal structure of AAC(6′)-I_i in complex with AcCoA,⁴⁵ which reveals two hydroxyl groups, Tyr147-OH (3.71 Å away) and Thr111-OH (4.31 Å away), near the sulfur atom of CoA. It was hypothesized that oxidation of the sulfur atom to either a sulfoxide or a sulfone, might increase affinity for the enzyme by allowing two extra H-bonds between the oxygen of S=O and the two hydroxyl groups. One pot chemoselective oxidation of **7a-b** to sulfoxides **10a-b**, or to sulfones **11a-b** was achieved in water using ammonium persulfate or oxone, respectively (Figure 4B). The results of AAC(6′)-I_i inhibition studies reveal that two of these compounds show nanomolar activity (**11a** and **11b**). The low micromolar activity observed for the other two suggests that correct

positioning of the S=O bond matters for interaction with Tyr147-OH and Thr111-OH, and/or to prevent clashes with the enzyme.¹⁰⁹

4.1.4 Modifications to the aminoglycoside moiety of the bisubstrates—

AAC(6')s are known to be highly promiscuous and acetylate not only a variety of aminoglycosides, but also selected peptides and proteins. The effect of varying the aminoglycoside part on the activity of bisubstrates was investigated with compounds **12** (neamine replaced with the 4,6-linked aminoglycoside kanamycin A) and **13** (neamine replaced with the 4,5-linked aminoglycoside ribostamycin).¹⁰⁷ The K_i values reported are similar for all three analogs **7a**, **12** and **13** (Figure 4C), which suggests that aminoglycoside rings III and IV are not necessary for inhibition. Studies with compound **14** which lacks ring II (the 2-deoxystreptamine or 2-DOS ring) on the other hand imply that ring II is essential for activity of the bisubstrates.¹⁰⁸ From these results, rings I and II (neamine) are essential and sufficient for nanomolar inhibition of AAC(6')-Ii by bisubstrates.

Poly-L-lysines are known substrates of AAC(6')-Ii that are acetylated at one ϵ -NH₂.⁵⁷ This activity is consistent with the reported activity of AAC(6')-Ii on some positively charged proteins such as histones. Replacement of the aminoglycoside moiety of **7a** with a N^α-acetyl-L-lysine methyl ester (**15**), led the K_i to increase by more than 2 orders of magnitude.¹⁰⁸ The lack of positive charges on the lysine derivative may explain this result. To validate this hypothesis, substitution with arginine (**16**) was achieved, and resulted in one order of magnitude loss of activity compared to **7a**, which is consistent with the beneficial effect of adding a positive charge.¹⁰⁸ Results with propylenediamine (reported to imitate the diamine portion of 2-DOS in aminoglycosides⁹²) derivative **17** are also in line with this trend.¹⁰⁸ Synthesis of more rigid piperazine derivatives **18a** and **18b** aimed at eliminating entropic costs possibly incurred upon binding to the enzyme.¹⁰⁸ K_i values for **18a-b** are similar to those of **16** and **17** (Figure 4C). Overall the above data suggest that for optimal activity, bisubstrates should either include both rings I and II of an aminoglycoside, or positive charges properly oriented to mimic the substrate. Although highly potent inhibitors, the bisubstrates discussed so far are not active in cellular assays, likely due to their negatively charged groups which may prevent cell permeability.

4.1.5 Modifications to the adenosine diphosphate moiety of bisubstrates—

Because of its multiple negative charges, the adenosine diphosphate moiety is likely responsible for preventing the bisubstrates from penetrating cells. Compounds **19a-e** (Figure 4D) which are bisubstrate analogs lacking the adenosine diphosphate group, were therefore synthesized but reported to show no significant inhibitory activity against AAC(6')-Ii (up to 500 μ M).^{108, 112} On the other hand, compound **20** which lacks the adenosine monophosphate moiety but retains one phosphate group, shows low micromolar activity. Analysis of the AAC(6')-Ii crystal structures reveals 5 potential hydrogen bonding interactions between this phosphate groups and the enzyme.⁴⁵⁻⁴⁷ Although beneficial to inhibitor potency, the negative charge of the phosphate group on compound **20** suffices to compromise cell-based activity. Analogues containing mimics of the diphosphate moiety, such as compounds **21a-b**, **22a-b** and **23a-c** were also reported (Figure 4D).^{108, 111} It was found that although succinate (see **21b**) was a poor mimic of the diphosphate group,

malonate (see **21a**) and acetoacetate (see **22a**) proved to be very good replacements. Even though the *in vitro* AAC(6′)-Ii inhibition activity of the last two compounds is similar, their potentiation effect on the activity of kanamycin A against a resistant strain of *E. faecium*, differ largely, and only **22a** was found to be biologically active.¹⁰⁸ It is hypothesized that the negative charge of the carboxylate group of **21a** may be detrimental to activity.

Compound **22b** was also reported with the aim of replacing the ester bond of **22a** with a more inert amide bond.¹¹¹ AAC(6′)-Ii inhibition studies showed that **22b** was ~20 fold less potent than **22a**. This loss of inhibitory activity was unexpected and rationalized based on the different geometry of amides and esters, which may affect the orientation of the acetoacetate group in the enzyme active site. Even the addition of substituents expected to reach into potential hydrophobic binding pockets (see compounds **23a-c**) was not sufficient to counter the negative effect of the amide bond.¹¹¹

These studies highlight the important contribution of the diphosphate moiety on AAC(6′) inhibition by bisubstrates. Attempts to replace the diphosphate with non-charged mimics have nevertheless produced active molecules, one of which (**22a**) is the first biologically active molecule in blocking aminoglycoside resistance caused by expression of AAC(6′) in cells. The *in vitro* inhibitory effect of **22a** on AAC(6′) is, however, considerably reduced in comparison to the most potent bisubstrates.

4.1.6 Production of bisubstrate inhibitors by the bacteria themselves—To capitalize on the potency of the bisubstrate inhibitors while addressing the issue of cell membrane penetrability, a prodrug strategy has also been exploited.¹¹² As mentioned above, aminoglycoside-pantetheine analogues **19a-e** have no detectable effect on the activity of AAC(6′)-Ii. Many pantetheine derivatives are known to be transformed by the CoA biosynthetic enzymes to the corresponding CoA-containing analogues.^{128–130} With this in mind, it was envisioned that membrane-permeable compounds **19a-e** could be transformed within bacteria by the enzymes pantothenate kinase (PanK), phosphopantetheine adenylyltransferase (PPAT), and dephosphocoenzyme A kinase (DPCK) into the bisubstrates **7a-e** (Figure 6). The ability of compounds **19a-e** to be transformed by these enzymes was confirmed by both *in vitro* enzyme studies with the *E. coli* PanK, PPAT and DPCK enzymes, and in cells with *E. faecium*. Derivatives with aminoglycosides other than neamine have shown similar activity. To date, compounds **19b-e** are the most potent molecules shown to block resistance and lower the MIC of kanamycin A against a resistant strain.

4.1.7 Fragment-based design—NMR-guided fragment screening has also had success in identifying inhibitors of AAC(6′)-Ib. Recognizing the importance of the 2-deoxystreptamine (2-DOS) ring of aminoglycosides, it was rationalized that compound **24a** (Figure 7) would act as a good 2-DOS analogue due to its (1) constrained cyclic structure, (2) pair of amino groups with similar geometry to those of 2-DOS, and (3) presence of a hydroxyl group. Moreover compound **24a** is synthetically more accessible than 2-DOS. Using saturation transfer difference (STD) and reverse NOE pumping experiments, compound **24a-d** were determined to be ligands of AAC(6′)-Ib, and subsequent titration experiments yielded K_d values in the range of 20–60 μ M.¹¹³ Next **24d** was linked to CoA to

afford the bisubstrate-like inhibitor **25**, which displays a K_i of 500 nM against AAC(6')-Ib.
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4.2 Regulating AAC(6') protein expression

Antisense oligonucleotides have been successfully used to block expression of the gene encoding for AAC(6')-Ib. RNase H mapping and computer prediction identified five single stranded regions on the *aac(6')-Ib* mRNA that were potentially accessible for interaction with antisense oligonucleotides.¹³¹ To confirm the viability of these target regions, various complimentary oligodeoxynucleotides were synthesized and tested *in vitro* for their effect on RNase H digestion. Electroporation of the bacterial cells was required for introduction of these oligonucleotides into the cell. As an alternative strategy, the authors next turned to external guide sequences (EGSs), which are short antisense oligoribonucleotides that induce RNase P-mediated cleavage of a target mRNA. A total of 39 different EGSs were designed to target the aforementioned five regions accessible for interaction on the *aac(6')-Ib* mRNA.¹³² An *E. coli* strain harboring the *aac(6')-Ib* gene was then transformed with recombinant plasmids coding for selected EGSs, and subsequently tested for resistance to amikacin. The EGS with the highest *in vitro* mRNA binding affinity, EGSC3 (caaguacuguuccacca, with lower cases implying RNA), was able to significantly slow the growth of *aac(6')-Ib* harboring *E. coli*. Since nucleases rapidly degrade oligoribonucleotides, nuclease-resistant oligonucleotides were next pursued. Using EGSC3 as a basis, a series of locked nucleic acid (LNA)/DNA co-oligomers were synthesized.¹³³ LNA9 (CAAGTACTGTTCCACCA, with DNA in upper cases and LNA underlined) was the most effective and caused significant growth inhibition at concentrations of 50 nM. LNA9 is also stable to nucleases as incubation studies with cell cultures and extracts confirms its presence even after 24 hours.

5. SUMMARY AND CONCLUSIONS

Roughly half of the antibiotics that are clinically used today were discovered in the “golden age” of antibiotics, i.e. from 1950 to 1960.¹³⁴ At the time, the confidence on the impact that antibiotics would have on human health was so high, that in 1969 William Stewart, then US Surgeon General, told the US Congress that “It’s time to close the book on infectious diseases, and declare the war against pestilence won...”. Unfortunately, 40 years later, the war is still on. Although bacterial resistance to antibiotics did exist at the time, the sheer volume of new antibacterial discoveries was enough to mask the underlying problem at hand. Following the discovery of carbapenems in 1976; however, only 3 antibiotics from new structural classes have been brought to clinics for human use: the narrow spectrum drugs linezolid, daptomycin, and retapamulin. Challenges also lie in the stringent requirements set by the health agencies, and in the current economic realities that ward off pharmaceutical companies from engaging in antibacterial research and discovery programs.
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Multiple strategies are necessary to combat antibiotic resistance. The search for new antibacterial agents (including drug combinations) remains essential, yet other promising strategies include the development of drugs targeting resistance-causing mechanisms, and agents that affect the host’s immune system or bacterial virulence. Resensitization of

resistant strains to existing antibiotics is an established concept. Although numerous resistance mechanisms have been reported for penicillins (efflux, decreased cell permeability, and expression of various β -lactamases), the combination of a penicillin with a β -lactamase inhibitor has proven useful clinically and has generated billions of dollars in profit in one year only. This tactic not only provides health care providers with an alternative option to treat resistant infections, but it may also prolong the time required for bacteria to gain further resistance. In spite of the breadth of work accomplished so far in developing inhibitors against AMEs, many questions are still left unanswered. More information about the mechanism of AACs, APHs, and ANTs will facilitate the design of antibiotics that escape their action and the discovery of new resistance inhibitors.

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Figure 1.

Typical product of aminoglycoside acetylation by AAC(6') and the stereo- and electrostatic effects that this modification has on the interaction between the aminoglycoside 6'-NH₂ and A1408 of the 16S rRNA. Shown is the 4,5-disubstituted deoxystreptamine aminoglycoside, ribostamycin. Acetylation by AAC(6') is highlighted in red.

Figure 2.

A) Representation of the KNF model for a homodimeric protein.⁶⁸ Upon ligand (red square) binding, subunit conformation changes from binding-incompetent (blue triangle) to binding-competent (green square). If the state of $\Delta\Delta$ is energetically more favourable than $\square\square$, binding is negatively cooperative. If the state of $\Delta\Delta$ is energetically more favourable than $\square\square$, binding is positively cooperative. **B)** Representation of the HT model for a homodimeric protein.⁶⁷ Enzyme subunits exist in a dynamic equilibrium between folded (green square) and unfolded (green lines) states. Ligand (red square) can only bind to a folded subunit. Cooperativity is determined by the energetic coupling between the two subunits. In cases where G (the energy change associated with breaking the interface between two folded subunits) is positive, it is favourable for the two subunits to interact. As the complete folding of both subunits is required for the second binding event, this interaction reduces the total free energy of the system. This results in positive cooperativity. In the cases where G is negative, the reverse occurs. **C)** Representation of the model proposed for the interaction of AAC(6')-Li with AcCoA.⁶⁴ Intrinsic positive cooperativity of binding is observed for AcCoA; however, by affecting the folding/unfolding equilibria, the temperature determines whether overall positive or negative cooperativity is observed.

Figure 3. Selected aminoglycoside analogues that have been tested against AAC(6') enzymes as well as AAC(6')-producing bacterial strains. Biological data gathered for other AMEs and other AME-expressing strains are omitted.

A. BISUBSTRATES AND LINKER LENGTH

Compound	n	K _i (μM)
7a	1	0.076 ± 0.025
7b	2	0.043 ± 0.023
7c	3	0.161 ± 0.098
7d	4	8.0 ± 2.6
7e	5	5.2 ± 0.7

C. AMINOGLYCOSIDE MOIETY MODIFICATIONS

R	Compound	K _i (μM)
	12	0.111 ± 0.028
	13	0.119 ± 0.014
	14	3.4 ± 2.0
	15	~130
	16	1.2 ± 0.2
	17	3.4 ± 1.7
	18a (m = 1)	3.6 ± 0.4
	18b (m = 2)	7.4 ± 2.9

B. LINKER MOIETY MODIFICATIONS

R	Compound	K _i (μM)
	8	1.6 ± 0.6
	9	2.0 ± 0.9
	10a (n = 1)	0.06 ± 0.03
	10b (n = 2)	2.0 ± 0.7
	11a (n = 1)	0.27 ± 0.2
	11b (n = 2)	0.09 ± 0.06

D. ADENOSINE DIPHOSPHATE MOIETY MODIFICATIONS

R	Compound	n	K _i (μM)
	19a	1	≥ 500
	19b	2	≥ 500
	19c	3	≥ 500
	19d	4	≥ 500
	19e	5	≥ 500
	20	1	12 ± 8
	21a (m = 1)	1	2.2 ± 1.1
	21b (m = 2)	1	≥ 1000
	22a (X = O)	1	11 ± 6
	22b (X = NH)	1	≥ 200
	23a (m = 1)	1	≥ 200
	23b (m = 2)	1	≥ 200
	23c (m = 3)	1	≥ 200

Figure 4.

Structure and inhibition constants (K_i) for the AAC(6′)-Ii inhibitors discussed in this review.

Figure 5.
Tetrahedral intermediate postulated for AAC(6') catalysis

Figure 6.

Proposed mechanism for the enzymatic activation of **19a-e** to **7a-e** by the bacterial enzymes PanK, PPAT, and DPCK.

Figure 7.
Small, micromolar ligands **24a-d**, and nanomolar inhibitor of AAC(6')-Ib, compound **25**.

Table 1

Determination of IC₅₀ values for various cationic antimicrobial peptides and their derivatives against AAC(6')-Ii and AAC(6')-APH(2'')

Peptide	Sequence	Charge at pH 7	IC ₅₀ (μM) ^a	
			AAC(6')-Ii	AAC(6')-Ie ^b
PG1	RGGRLCYCRRRFCVCVGR	+6	n.a. ^c	n.a.
Gramicidin S	(cyclic) LP ^d PVOLFPVO	+2	n.a.	14 ± 2
CP29	KWKSFIKKLTAVKKVLTGLPALIS	+6	n.a.	21 ± 9
CP2600	KWKSFIKKLTSAKKVTTAAKPLTK	+7	n.a.	38 ± 9
CM3	KWKKFIKSLTKAAKTVVKTAKKPLIV	+9	24 ± 1	n.a.
Indolicidin	ILPWKPWWPWRR	+4	13 ± 1	n.a.
CP11CN	ILKKWPWWPWRRK	+6	23 ± 4	n.a.
CP10A	ILAWKWAWWAWRR	+4	4.4 ± 0.2	n.a.

^a determined with 50 μM acetyl CoA and 50 μM kanamycin A

^b AAC(6')-Ie is the isoform that constitutes AAC(6')-APH(2'')

^c n.a. = no activity