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REVIEW

A review of patents (2011-2015) towards combating resistance to and otoxicity of aminoglycosides

Nishad Thamban Chandrika[a] and Sylvie Garneau-Tsodikova[a],*

Since the discovery of the first aminoglycoside (AG), streptomycin, in 1943, these broad-spectrum antibiotics have been extensively used for the treatment of Gram-negative and Gram-positive bacterial infections. The inherent toxicity (ototoxicity and nephrotoxicity) associated with their long-term use as well as the emergence of resistant bacterial strains have limited their usage. Structural modifications of AGs by AG-modifying enzymes, reduced target affinity caused by ribosomal modification, and decrease in their cellular concentration by efflux pumps have resulted in resistance towards AGs. However, the last 10 decade has seen a renewed interest among the scientific community for AGs as exemplified by the recent influx of scientific articles and patents on their therapeutic use. In this review, we use a non-conventional approach to put forth this renaissance on AG development/application by summarizing all patents filed on AGs from 2011-2015 and highlighting some related publications on the most recent work done on AGs to overcome resistance and improving their therapeutic use while reducing ototoxicity and nephrotoxicity. We also present work towards developing amphiphilic AGs for use as fungicides as well as that towards repurposing existing AGs for potential newer applications.

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Introduction

Aminoglycosides (AGs) are broad-spectrum antibiotics commonly used for the treatment of serious bacterial infections.[1] AGs, which are naturally occurring and semi-synthetic amino-modified sugars, can be classified 20 based on their structure and clinical efficacy. Structurally, AGs can be classified based on the substitution pattern of their 2-deoxystreptamine (2-DOS) ring as, 4,5-disubstituted 2-DOS AGs (*e.g.*, neamine (NEA), neomycin B (NEO), paromomycin (PAR), and ribostamycin (RIB)), 4,6-disubstituted 2-DOS AGs (*e.g.*, amikacin (AMK), arbekacin (ABK), 25 dibekacin (DBK), gentamicin (GEN), geneticin (G418), kanamycin A and B (KANA and KANB), sisomicin (SIS), tobramycin (TOB), and plazomicin (PLZ)), and other monosubstituted 2-DOS AG scaffolds (*e.g.*, apramycin (APR) and streptomycin (STR)) (Fig. 1). Clinically, AGs are used systemically or topically for treating infections caused by Gram-30 positive and Gram-negative bacterial strains. The AGs AMK, GEN, KANA, NEO, PAR, STR, and TOB are currently approved by the US Food and Drug Administration (FDA) and available for clinical use in the United States. The most common clinical applications of AGs are either alone or as part of combination therapy in the treatment of serious 35 infections caused by aerobic Gram-negative bacilli, while less common is their application in combination with other agents against select Gram-positive pathogens.[2-7] In addition, certain AGs have been found to display clinically relevant activity against protozoa (PAR), *Neisseria gonorrhoeae* (STR), and mycobacterial infections (AMK, ABK, KANA, 40 STR, and TOB). The recent emergence of infections due to Gram-negative bacteria and enhanced bacterial resistance have prompted a revived interest in the use of AGs while addressing the bacterial resistance, nephrotoxicity, and ototoxicity problems associated with their clinical use.

45   As with any antibiotics, bacterial resistance has also contributed to the problems associated with AGs.[8] The most prevalent modes of action leading to AG bacterial resistance are (i) reduction in the intracellular

concentration of antibiotics by efflux pumps or through reduced membrane permeability, (ii) structural modification of the 16S ribosomal 50 RNA (rRNA) that lead to reduced target affinity, and (iii) structural modification of AGs by AG-modifying enzymes (AMEs). The AMEs, which include AG *N*-acetyltransferases (AACs), AG *O*-phosphotransferases (APHs), and AG *O*-nucleotidyltransferases (ANTs) act by chemically modifying the AGs, which adversely affects their 55 clinical efficacy.[9] AACs catalyze the acetyl coenzyme A-dependent acetylation of amine groups, while APHs and ANTs catalyze the ATP and/or GTP-dependent phosphorylation and nucleotidylation of hydroxyl groups of AGs, respectively.[10-12] The above classes of AMEs are typically monofunctional enzymes, but the emergence of bifunctional enzymes is 60 another aspect relevant to the clinical use of AGs. Four genes encoding bifunctional AMEs have been identified so far: AAC(6')-Ie/APH(2'')-Ia from *Staphylococcus aureus*,[13-16] ANT(3'')-Ii/AAC(6')-IId from *Serratia marcescens*,[17-19] AAC(3)-Ib/AAC(6')-Ib' from *Pseudomonas aeruginosa*,[20, 21] and AAC(6')-30/AAC(6')-Ib from *P. aeruginosa*.[22, 23] 65 Unlike other regiospecific AMEs, the newly discovered enhanced intracellular survival (Eis) protein found in *Mycobacterium tuberculosis* as well as other mycobacterial and non-mycobacterial strains is a versatile enzyme that can multiacetylate different amine positions of AGs.[24-35]

    To overcome antibiotic resistance, various strategies are currently 70 employed: (i) development of newer AG scaffolds; (ii) derivatization of existing FDA approved AGs, and (iii) repurposing existing AGs for new applications.[36] In order to create more potent and less toxic AGs capable of evading resistance mechanisms, several approaches can be used, including chemical and enzymatic modification of hydroxyl and amine 75 groups at different positions of AG scaffolds, designing structurally constrained AGs, coupling of AGs to form AG dimers, AG-lipid conjugates, and AG-drug conjugates by synthetic or semi-synthetic methods.[37]

    To highlight the great importance of this old class of antibiotics and 80 the regain in interest for their usage and development, herein, we decided to write a review in a non-traditional fashion by focusing on the multiple

patents (19 of them!) on AGs that appeared in the last four years alone. In this review, all recent patents (filed from 2011-2015) covering synthetic modifications of AGs as well as current and potential new applications of AGs as antibacterial (15 patents), antifungal (2 patents), and antiviral (1 patent) agents, as well as their use as therapeutics for genetic disorders (1 patent) will be discussed along with scientific literature related to these patents. The main focus of these patents is on the work done to overcome resistance and increasing the potency and spectrum of activity of these AGs, thus improving their therapeutic index by decreasing their nephrotoxicity and ototoxicity.
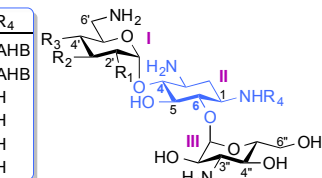

Fig. 1. Structures of AGs presented in this review.

Aminoglycosides as antibacterial agents

AGs exert their antibacterial activity by binding to bacterial ribosomes (helix 44 (h44) of the 16S rRNA or to the major groove of helix 69 (H69) of the 23S rRNA of the 50S subunit), thereby preventing the initiation of the complex process of protein synthesis.[38-42] Examples of usage of AGs include: STR, which has been used extensively as a primary drug in the treatment of tuberculosis; GEN, which is active against multiple strains of Gram-positive and Gram-negative bacteria, including *P. aeruginosa*; KAN, which is active at low concentrations against many Gram-positive

bacteria including penicillin-resistant *Staphylococci*; as well as GEN and TOB, which are mainstays for the treatment of *Pseudomonas* infections. With the emergence of multidrug-resistant (MDR) bacterial strains (and extensively-drug resistant (XDR), in the case of *M. tuberculosis*), many of the currently marketed AGs are not effective against numerous of these bacterial infections. There is therefore an urgent need for new and improved AG antibiotics. In order to circumvent the problems associated with antibacterial susceptibility and toxicity, extensive research was performed and is still underway to improve the bactericidal activity of AGs. This section on AGs as antibacterial agents will focus on the challenges associated with their chemical synthesis, with emphasis on (i) developing single-step derivatization as well as stereo- and regioselective modifications of AGs, (ii) r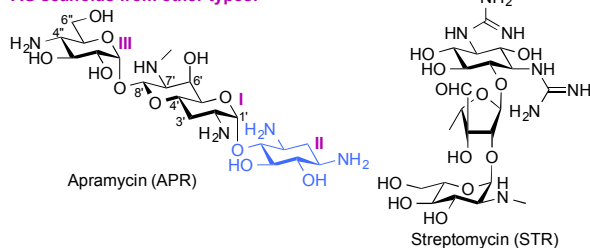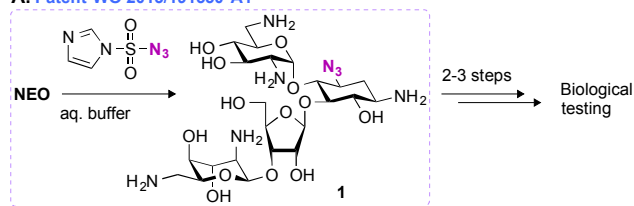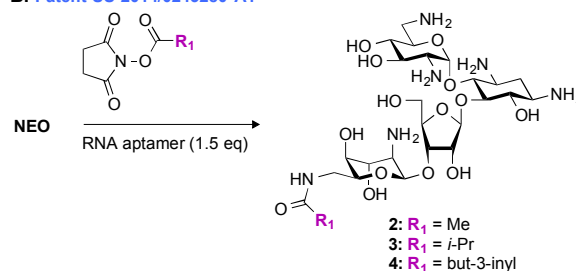educing the number of synthetic steps involved in formation of AG scaffolds, and (iii) designing AG scaffolds based on their mechanism(s) of action in bacteria and/or aimed at improving their antibacterial activity, overcoming the action of resistance enzymes, and reducing the toxicity associated with their usage.
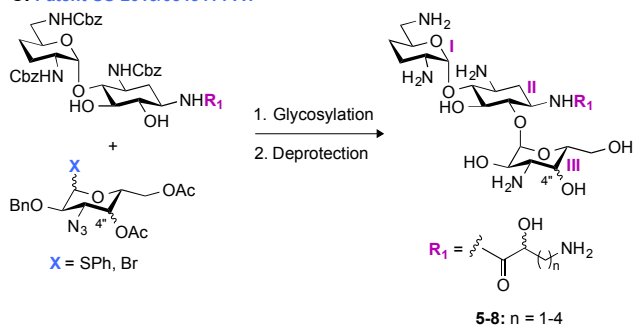
Fig. 2. Representative scheme for the **A.** single-step C3-azide derivatization of AGs, **B.** single-step 6'''-amide derivativation of AGs using RNA aptamers, and **C.** multistep synthesis of ABK derivatives from carbohydrate building blocks.

Patent WO 2013/191550 A1[43]

Most regioselective modifications of AGs require multistep chemical syntheses involving tedious and cost ineffective functional group protection/deprotection manipulations due to presence of numerous hydroxyl and amine groups with similar reactivity on AG scaffolds.[44-48] In contrast, a more facile introduction of functionalities in structurally complex molecules generally depends on non-covalent protecting group

strategies[49, 50] and enzymatic approaches.[51]

In patent WO 2013/191550 A1,[43] a novel one-step regioselective chemical diazotation of the C3-amine functionality of the 2-DOS ring is presented (Fig. 2A). This selective diazotation of AGs employs imidazole-1-sulfonyl azide, a shelf-stable, non-explosive, and water-soluble reagent.[52] This method is cost efficient, scalable, and can be performed at neutral conditions without requiring extensive protection and deprotection chemistry. This methodology was proven successful by modifying the AGs AMK, APR, PAR, RIB, NEA, and NEO. To demonstrate the applicability of this new synthetic transformation in generating useful AG molecules, the C3-azido-NEO derivative **1** produced was further modified in 2-3 steps, and the resulting compounds were tested against a NEO-sensitive *Escherichia coli* strain and its resistant counterpart containing an AAC(3) enzyme (Fig. 2A). The C3-NEO derivatives were found to be active against the resistant strain, demonstrating that modification at the C3-position can overcome the action of the AAC(3) resistance enzyme. A similar observation was previously reported for AGs modified by AAC(3)-IV that were found to resist the action of AAC(3) and AAC(6') enzymes.[53] Selective transformation of the amino group at the 3-position of ring I of AGs into an azide, followed by azide modification into a moiety that cannot be *N*-acetylated by AAC is therefore a promising tool to protect AGs against AMEs.

Patent US 2014/0243280 A1[54]

An alternative method for derivatizing AGs in one regioselective chemical reaction is highlighted in patent US 2014/0243280 A1 (Fig. 2B).[54] The approach presented relies on the use of non-covalent supramolecular protecting groups (SPGs) based on host-guest interaction and offers a new and appealing perspective for single-step modifications of AGs. Prior to this patent, calixarenes had been reported as non-covalent protecting groups.[55] However, due to the small size of the calixarenes' cavity, their use was limited to modifications of small molecules containing a maximum of two chemically equivalent functional groups. In order to overcome this drawback and to generalize this concept to AGs, in patent US 2014/0243280 A1, a novel class of SPGs based on oligonucleotides[56-58] like Apt1 (23mer) and Apt2 (21mer) was developed. These RNA aptamers (1.5 equivalent) in conjunction with *N*-hydroxysuccinimide esters (methyl, *iso*-propyl, or but-3-inyl) were efficiently used for the highly chemoselective (6'''-position) and regioselective (89 to >99%) transformation of AGs in very good yields (59-83%) (Fig. 2B). The efficacy of the compounds generated (**2-4**) was determined against one *E. coli* strain, and both NEO and PAR derivatives were found to display MIC values (6.3 μM and 12.5 μM for NEO and PAR derivatives, respectively) similar to that of their corresponding parent AGs. As the oligonucleotide-based SPGs could potentially modify a variety of AG scaffolds in one chemical step, this method could present an effective alternative for their rapid derivatization towards combating bacterial resistance. However, although single-step modifications show promise in derivatizing the 3- and 6'''-positions of some AGs, modifications at other positions still dependent on multistep synthetic routes.

Patent US 2013/0345411 A1[59]

In patent US 2013/03455411 A1,[59] a mutlistep strategy involving the use of basic carbohydrate building blocks and a glycosylation reaction between the 1''-position of ring III and the O6 of ring II to develop novel ABK analogues is presented (Fig. 2C). Building AG analogues by using

glycosylation has obviously been previously reported.[60-65] In this patent, by using this method, twelve ABK analogues (**5-8**) were synthesized and eleven of them were tested for their antibacterial activity against nine strains of MRSA. These ABK derivatives contained a hydroxyl moiety at the 4''-position oriented either axially or equatorially (*Note*: this orientation was introduced prior to glycosylation) as well as various side-chains ((*S*) or (*R*)-3-amino-2-propionate, (*S*) or (*R*)-AHB, (*S*) or (*R*)-5-amino-2-valerate, and (*S*) or (*R*)-6-amino-2-hexanoate) at the N1-position. The MIC values for these compounds were found to be 0.5-2 μg/mL (4-8 μg/mL for ABK) and 2-32 μg/mL (128 μg/mL for ABK) against ABK-sensitive and ABK-resistant MRSA, respectively. By measuring the concentration of *N*-acetylglucosamine in mouse urine, these compounds were found to display reduced nephrotoxicity (74.7 mIU) when compared to ABK (135.0 mIU).

In the past 20 years, derivatives of ABK, originally synthesized from DBK in 1973, have also been generated *via* other chemical approaches. Synthetic 2''-amino-2''-deoxy-ABK and its 5-epiamino analogue were prepared and found to display excellent antibacterial activity against MRSA.[66] The enzymatic modification of ABK by an *N*-acetyltransferase from *Streptomyces griseus* resulted in 3''-*N*-acetyl-ABK, but unfortunately this compound displayed 2 to 10-fold lower activity than ABK against 59 Gram-positive and Gram-negative bacterial strains.[67] The substitution of the 5-hydroxyl group of ABK by an amine moiety resulted in an analogue, which was superior to ABK against MRSA.[68] Synthetic 4''-deoxy-4''-episubstituted ABK and 4''-epi-5-deoxy-5-episubstituted ABK derivatives were also prepared, and among them 5,4''-di-epi-ABK was found to be a better antibacterial agent than ABK or 5-episubstituted ABK.[69] Finally, the 4''- and 6''-hydroxyl groups of ABK were regioselectively modified and 6''-amino-6''-*N*-[(*S*)-4-amino-2-hydroxybutyryl]-6''-deoxy-ABK was found to display excellent antibacterial activity, even better than that ABK.[70]

Patent WO 2011/143497 A1[71]

A common multistep approach for derivatizing AGs consists, as previously stated, of decorating parent AG scaffolds. In patent WO 2011/143497 A1,[71] 140 mono- and difunctionalized derivatives of GEN were synthesized by modifying its N1- and/or N6'-positions (Fig. 3A). These compounds were tested against one representative bacterial strain of each *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Acinetobacter baumannii*, which altogether contained AMEs that modify the N3- and O2''-positions of GEN. It was observed that 1,6'-disubstitution was preferable to overcome the action of AAC(6') enzymes in *E. coli*, *S. aureus*, *P. aeruginosa*, and *A. baumannii*. Overall, the two best GEN analogues identified were compounds **9** and **10** both containing a (*S*)-3-amino-2-hydroxypropynyl moiety at the 1-position and a (*R*)- or (*S*)-propane-1,2-diol at the 6'-position, respectively. In general, compound **9** was frequently found to be 2 to 4-fold more active than **10**. These compounds displayed no nephrotoxicity after 14 days at doses of 60 and 30 mg/kg when tested in rats. When tested for acute toxicity and cytotoxicity, compound **9** was found to present less acute toxicity than **10**, and cytotoxicity studies in HK-2 cells showed compound **10** to be at least 2-fold more cytotoxic than **9**.

Patent US 2013/0203693 A1[72]

Following up on the work done on derivatizing GEN in patent WO 2011/143497 A1,[71] further investigations on decorating different positions of other parent AGs were performed. In patent US 2013/0203693 A1,[72] structural modifications were made at the N1-, O5-, and O6''-positions of

TOB and AMK (Fig. 3B). In all cases, the final molecules contained a (*S*)-4-amino-2-hydroxybutyrate (AHB) group at the N1-position, whereas

A. Patent WO 2011/143497 A1

B. Patent US 2013/0203693 A1

C. Patent WO 2014/013495 A1

R$_1$ = C$_{12}$H$_{25}$, C$_{14}$H$_{29}$, C$_{16}$H$_{33}$
R$_2$ = C$_{12}$H$_{25}$, C$_{14}$H$_{29}$, C$_{16}$H$_{33}$
R$_3$ = C$_{12}$H$_{25}$, C$_{14}$H$_{29}$, C$_{16}$H$_{33}$
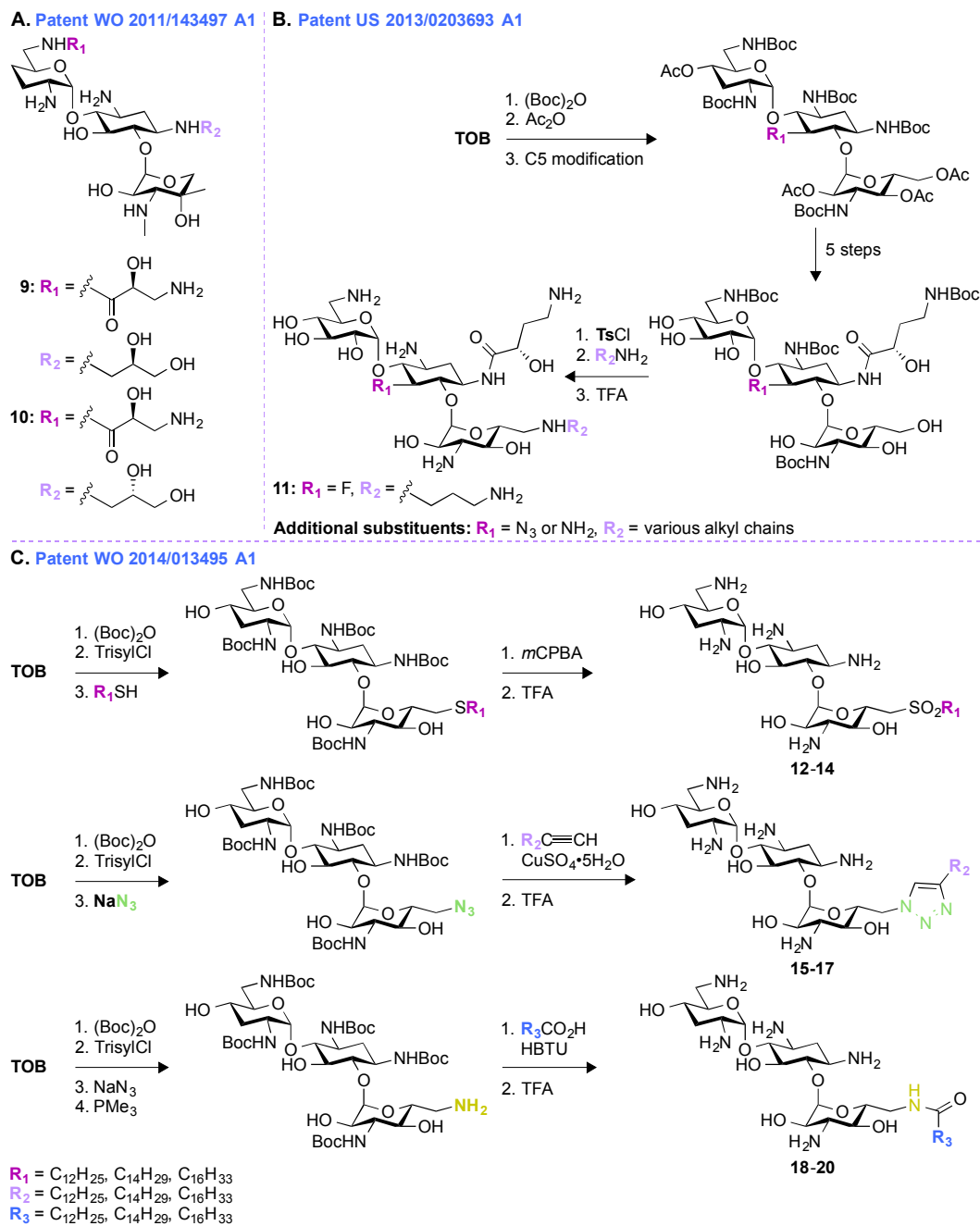
Similar derivatives were synthesized for KANA

Fig. 3. Representative scheme for the preparation of **A.** 1,6'-dimodified GEN derivatives, **B.** 1,5,6"-trisubstituted TOB derivatives (*Note*: 5,6"-disubstituted AMK derivatives were also similarly produced), and **C.** amphiphilic TOB derivatives with various linker types at the 6"-position.

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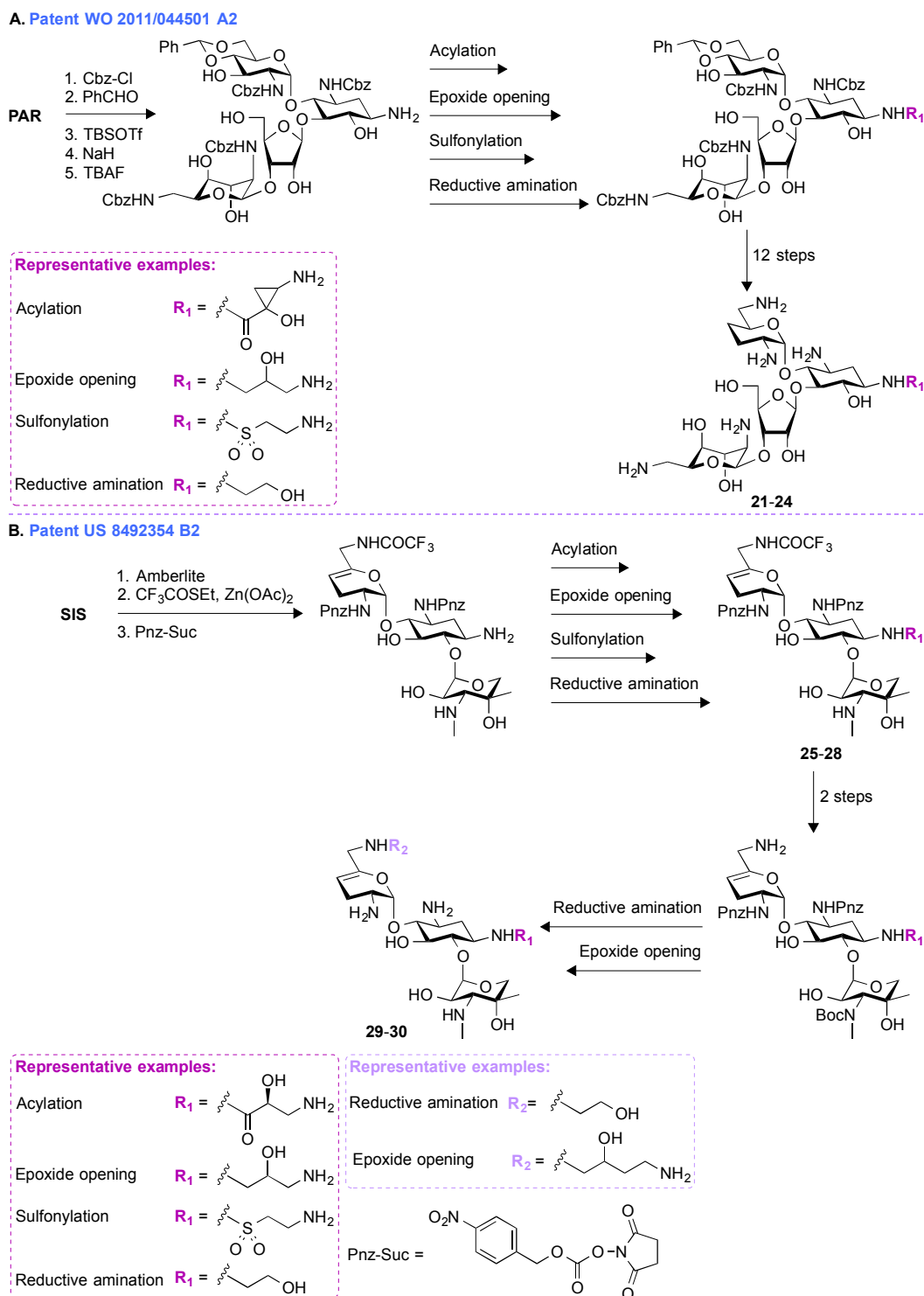**A. Patent WO 2011/044501 A2**

B. Patent US 8492354 B2

Fig. 4. Representative scheme for the preparation of **A.** NEO N1-substituted,3',4'-dideoxy-NEO analogues from PAR, and **B.** 1,6'-disubstituted SIS derivatives.

F, N_3, and NH_2 were found in place of the hydroxyl at the 5-position. Various substituents were introduced at the 6"-position by displacing a tosyl group with structurally diverse aliphatic amines. For evaluation of their clinical efficacy, these molecules were tested against a panel of Gram-negative bacterial strains including *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *A. baumannii*. As *P. aeruginosa* is a prominent nosocomial pathogen and has acquired multiple mechanisms of resistance, these compounds were tested against four strains of *P. aeruginosa* and MIC_{90} values were found to be in single digit compared to ≥ 64 µg/mL for the parent AGs. When tested against four strains of *K. pneumoniae* and *A. baumannii*, the structural modifications on these compounds were shown to result in an increased potency. Compound **11** was found to be most effective against AMK-resistant *P. aeruginosa* in an animal model. Overall, from this patent, it can be concluded that structural modifications at the N1-, O5-, and O6"-positions of TOB and AMK are beneficial.

Patent WO 2014/013495 A1[73]

To expand the scope of structural modifications of parent AGs, in patent WO 2014/013495 A1,[73] work was done to synthesize various cationic amphiphilic derivatives of TOB, KAN, and PAR without the use of an extensive number of synthetic steps to target the negatively-charged lipopolysaccharides present in bacterial cell wall through ionic interactions (Fig. 3C).[74, 75] Membrane-targeting AGs offer certain advantages over antibiotics targeting intracellular components as they are not dependent on bacterial cell cycle state, can evade intracellular resistance mechanism resulting in prolonged clinical efficacy, and do not display cell permeability issue.[76, 77] The sugar core, number of positive charges, length of aliphatic chains, and the type of chemical bond (thioether, sulfone (**12-14**), sulfonyl (**15-17**), amides, and triazolyl (**18-20**)) between the hydrophobic and hydrophilic parts of the molecules was taken into account while designing these compounds. These molecules were tested against eleven strains of Gram-positive and Gram-negative bacteria and MIC values for TOB against *S. pyogenes* M12 was found to be 64 µg/mL, whereas thioether, triazole, and amide derivatives were found to be 16 to 32-times more potent. The triazole derivative with a C_{12} chain (MIC 64 µg/mL) and its counterpart amide (MIC 32 µg/mL) showed improved potency compared to TOB (MIC 128 µg/mL). The minimal biofilm inhibition concentration (MBIC) for TOB and its synthetic derivatives were found to be 64-128 and 4-32 µg/mL, respectively. An hemolytic assay using erythrocytes from rats at concentrations 32, 64, and 128 µg/mL showed that there is no linear correlation between the antimicrobial potency and the hemolytic activity of the amphiphilic TOB derivatives. Overall, the optimal aliphatic chain lengths were found to be 12-16 and 4-8 carbons for mono- and disubstitution, respectively. More details about the results presented in patent WO 2014/013495 A1 can be found in the literature.[78-80]

Patent WO 2011/044501 A2[81]

The pharmaceutical company Achaogen was early on involved in multistep synthetic efforts towards derivatization of AGs like NEO. In patent WO 2011/044501 A2,[81] in order to accelerate the process of developing new antibiotics, a chemical strategy was developed for synthesizing various NEO analogues from PAR (Fig. 4A, **21-24**). A library of N1-substituted,3'-deoxy-NEO, N1-substituted,4'-deoxy-NEO, and N1-substituted,3',4'-dideoxy-NEO analogues was synthesized by using acylation with acids or activated esters, sulfonylation, reductive amination, or epoxide opening chemistry. The MIC values were

determined for a select group of analogues against one strain of *P. aeruginosa* and *E. coli* and were found to be ≤ 1 µg/mL and ≤ 1-16 µg/mL, respectively.

Patent US 849354 B2[82]

Later on, in addition to the NEO derivatives of patent WO 2011/044501 A2,[81] Achaogen was successful in generating the SIS derivative PLZ (formally known as ACHN-490), which was found to evade the resistance normally associated with AGs, and more importantly to display little to no toxicity.[83] In patent US 849354 B2,[82] various 1,6'-disubstituted (Fig. 4B, **29-30**, including PLZ) and 1,2'-disubstituted SIS derivatives were synthesized. Introduction of substituents at the 1-, 2'-, and 6'-positions was achieved by a combination of acylation (with acids or activated esters), epoxide opening, sulfonylation, and reductive amination chemistry.

After the development of the very promising PLZ, which is currently in phase 3 clinical trials, Achaogen published a number of studies describing the activities of this latest addition to the AG family of drugs. In brief, PLZ was evaluated against 3050 *E. coli* clinical isolates and 102 MDR strains of *K. pneumoniae*, including a subset of 25 strains producing *K. pneumoniae* carbapenemase (KPC) resistance enzymes. Against *K. pneumoniae*, MIC_{50} values for GEN, TOB, and AMK were 8, 32, and 2 µg/mL, respectively; whereas that of PLZ was 0.5 µg/mL. Against *E. coli*, MIC_{90} values for GEN, TOB, and AMK were 32, 8, and 4 µg/mL, respectively; whereas that of PLZ was 1 µg/mL.[84-86] PLZ was also evaluated against 493 MRSA isolates collected in 2009-2010 from 23 US hospitals and the MIC_{50} and MIC_{90} values found to be 1 and 2 µg/mL, respectively.[87] Synergy time-kill studies performed on 47 MRSA strains with different resistance phenotypes showed that combinations of sub-inhibitory concentrations of PLZ and daptomycin yielded synergy against 43/47 strains at 24 h, PLZ and ceftobiprole showed synergy in 17/47 strains, while 6/47 strains showed synergy for sub-inhibitory combinations of PLZ and linezolid.[88] PLZ was also found to be active against most *enterobacteriaceae* species at a concentration below 4 µg/mL as well as carbapenem-resistant *enterobacteriaceae* isolates (2 µg/mL) and was found to be effective against AG-resistant *staphylococci* (MIC_{90} = 2 µg/mL).[89, 90] Finally, PLZ was found to be moderately active against the nocosomal pathogens *A. baumannii* and *P. aeruginosa* with $MIC_{50/90}$ values of 8/16 µg/mL and 8/32 µg/mL, respectively.[91]

These promising *in vitro* results prompted the *in vivo* efficacy testing of PLZ in animals.[92] When tested in combination with cefepime, doripenem, imipenem, or piperacillin-tazobactam in a synergy time-kill analysis against 25 *P. aeruginosa* strains with different resistance phenotypes, synergy was observed in $\geq 70\%$ and $\geq 80\%$ of strains at 6 and 12 h, respectively, and in $\geq 68\%$ at 24 h.[93] The pharmacokinetic evaluation and safety monitoring of PLZ injection in healthy subjects showed no nephrotoxicity and ototoxicity in humans.[94]

Patent WO 2014/1454713 A2[95]

Despite their ototoxic and nephrotoxic effects, AGs remain some of the most widely used antibiotics worldwide. Various techniques such as modulating dosage plans and the use of free radical scavengers were employed as attempts to reduce the overall toxicity associated with AGs.[96] The unexpected discovery that AGs enter hair cells at high level by passing through specific ion channel prompted postulation of a similar mechanism of action in the case of kidney.[97] In patent WO 2014/1454713 A2,[95] a novel chemical synthetic approach was used to develop analogues of SIS for alleviating AG ototoxicity while preserving antibacterial

activity (Fig. 5A, **31-39**). The AGs were modified based on the notion that hindering AG entry *via* the hair cell MET channel would prevent ototoxicity.[98] Based on the biophysical properties of the MET channel where electrochemical gradients drive cations through the channel, it was presumed that conversion of the free amine to sulfonamides would result in decrease in net charge and in turn reduced ototoxicity.[99] Functional groups such as SO_2Me, SO_2Ph, and $C=OPh$ were introduced at the N1- and N3"-positions. MIC and minimal bactericidal concentration (MBC) values were determined for these SIS derivatives. Substitution by SO_2Me was more effective than either SO_2Ph or $C=OPh$, and compound **31** showed decreased toxicity against hair cells in *P. aeruginosa* species (22 times less ototoxic than SIS) and *S. aureus* species (18 times less ototoxic than SIS). Modification at the N3"-position abolished antibacterial activity of all six SIS derivatives.
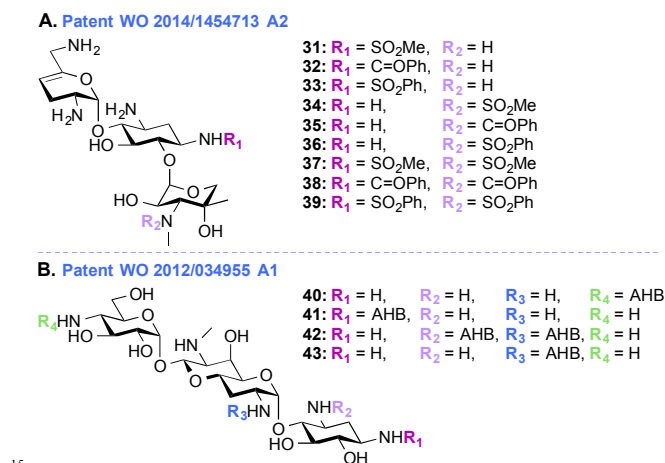

A. Patent WO 2014/1454713 A2

31: R_1 = SO_2Me, R_2 = H
32: R_1 = $C=OPh$, R_2 = H
33: R_1 = SO_2Ph, R_2 = H
34: R_1 = H, R_2 = SO_2Me
35: R_1 = H, R_2 = $C=OPh$
36: R_1 = H, R_2 = SO_2Ph
37: R_1 = SO_2Me, R_2 = SO_2Me
38: R_1 = $C=OPh$, R_2 = $C=OPh$
39: R_1 = SO_2Ph, R_2 = SO_2Ph

B. Patent WO 2012/034955 A1

40: R_1 = H, R_2 = H, R_3 = H, R_4 = AHB
41: R_1 = AHB, R_2 = H, R_3 = H, R_4 = H
42: R_1 = H, R_2 = AHB, R_3 = AHB, R_4 = H
43: R_1 = H, R_2 = H, R_3 = AHB, R_4 = H

Fig. 5. Representative structures of **A.** 1,3"-disubstituted SIS derivatives, and **B.** APR derivatives.

Patent WO 2013/170985 A1[100]

APR, an AG that has so far only been used in veterinary medicine, has more recently been explored as it was found to display reduced ototoxicity. AGs affect the protein synthesis through binding to specific sites of the rRNA and ring I of the 2-DOS derivatives binds to the rRNA through specific and nonspecific interactions irrespective of the 4,5- or 4,6-substitution pattern. Recent evidence implies mitochondrial protein synthesis as a key element in AG ototoxicity, and the defective mitochondrial function leads to reactive oxygen species (ROS) generation resulting in ototoxicity.[101, 102] In patent WO 2013/170985 A1,[100] the activities and ototoxicity of various parent AGs (depicted in Fig. 1), with a special focus on APR, is presented. APR, GEN, TOB, and KAN were tested for ribosomal specificity and were found to be similarly active towards bacterial ribosomes (IC_{50} 0.02-0.08 μM) and eukaryotic cytosolic ribosomes (IC_{50} cytosolic hybrids 40.4-91.2 μM). In contrast, when compared to 4,6-disubstituted AGs, APR displayed a 4-fold difference in IC_{50} values in the case of mitochondrial ribosomes (115.6 μM for APR *versus* 11.1-32.6 μM for 4,6-disubstituted AGs) and 50-fold difference in the case of mitohybrid ribosomes with mutation at A-site (47.5 μM for APR *versus* 0.69-1.12 μM for 4,6-disubstituted AGs). Crystallographic studies showed that the bicyclic ring II of APR stacks well to the guanine base of the C1409-G1491 base pair compared to the flexible monocyclic ring I of 4,6-disubstituted derivatives. *In vitro* and *in vivo* toxicity studies of AGs were carried out to explore the damage to the cochlea in both human and experimental animals. The dose response curve showed that 50% of all hair cells in the base of the cochlea were destroyed at 0.2 mM GEN, and complete elimination of outer hair cell was observed at 0.5 mM. In contrast, APR at a dose of 0.2 mM did not adversely affect cells even in the base, but was shown to affect basal hair cells at 2 mM concentration.

Patent WO 2012/034955 A1[103]

In addition to the work done on APR in patent WO 2013/170985 A1,[103] APR derivatives were developed towards treatment of human infections (Fig. 5B, **40-43**). In the past, APR has not been used in human because it was thought to have relatively high toxicity. However, work is currently underway to explore the use of APR derivatives as antibiotics (*e.g.*, acylated and alkylated derivatives of APR have been tested for their broad spectrum activity).[104] In patent WO 2012/034955 A1,[103] APR and its derivatives were tested against various Gram-negative bacterial strains and their activities were compared to those of parent AGs such as TOB, GEN, NEO, and PAR. More specifically APR was tested against clinical isolates of MRSA and its MIC values were found to be similar to those of TOB, SIS, KANA, AMK, NEO, and PAR. APR was also found to display affinity for wild-type bacterial ribosomes similar to those of TOB, NEO, and GEN, but the APR affinity for hybrid ribosomes was reduced when compared to these drugs. In *in vitro* cochleatoxicity studies, APR was found to cause <5% hair loss in guinea pigs. After publication of this patent, Crich and co-workers synthesized various O6'- and N7'-analogues of APR, which were screened for their ability to inhibit protein synthesis in cell-free translation assays. All derivatives prepared were less active than APR, which emphasized the role of the 6'-hydroxyl group and its configuration for APR activity.[105]

Patent WO 2012/097454 A1[106]

In addition to the general synthetic approaches for derivatizing AGs, conjugation of AGs with other biologically relevant molecules or drugs has been explored as an interesting alternative strategy for the development of newer AGs.[107-111] Nanomolar bisubstrate inhibitors where an AG is conjugated to coenzyme A (CoA) were reported.[112, 113] Structure-activity relationship (SAR) studies of truncated CoA bisubstrate revealed that the pantetheinyl group is not necessary for recognition by the enzyme, but at least one phosphate group is needed.[114] Following up with amide-linked analogues, various sulfonamide-, sulfoxide-, and sulfone-containing bisubstrate inhibitors were synthesized and found to be 40-fold less potent against AAC(6')-Ii when compared to their amide-linked counterparts.[115] A similar trend was observed for phosphonate-linked AGs and the decreased inhibitory potency was explained by proximity catalysis by enzymes.[116] AG-CoA bisubstrates are highly potent AAC(6') inhibitors, however their inability to penetrate cells preclude *in vivo* studies.

In patent WO 2012/097454 A1,[106] a class of AAC inhibitors comprised of pantetheine conjugated to the 6'-amine of AGs has been investigated (Fig. 6A, **44-49**). Among all the AMEs, AAC(6')s are some of the most frequently observed in clinical isolates. There is therefore an urgent need for compounds capable of inhibiting AAC(6')s and reversing the bacterial resistance associated with these enzymes. NEA, RIB, and KANA conjugated to the pantetheinyl arm of CoA were found to undergo intracellular biotransformation by pantothenate kinase (PanK) prior to inhibiting AAC(6') enzymes. When tested against an AG-resistant *Enterococcus faecium* strain, two of these conjugates (**48** and **49**) were found to partially resensitize the strain to KANA and TOB.[117]

Patent US 8809286 B2[118]

Besides conjugating AGs to CoA, as described above, in patent US 8809286 B2,[118] work was done to conjugate NEO and KANA to the FDA approved fluoroquinolone ciprofloxacin (CIP) by using click chemistry
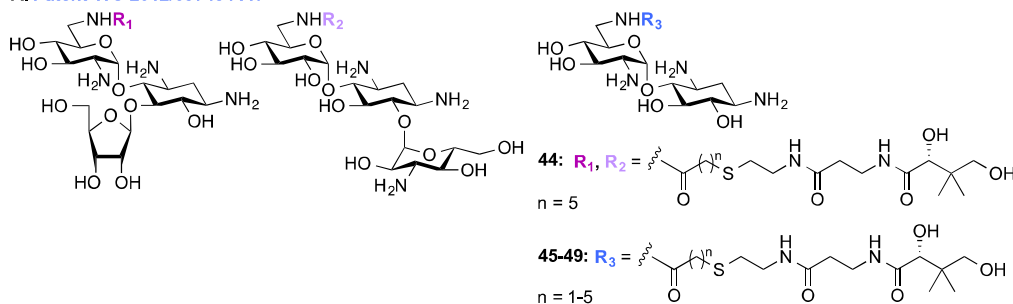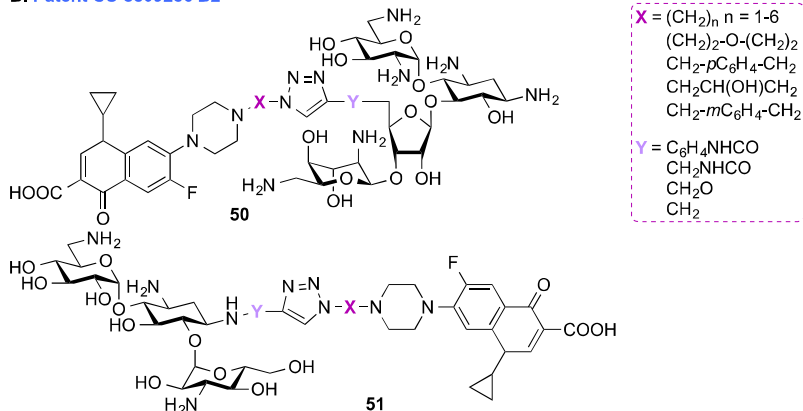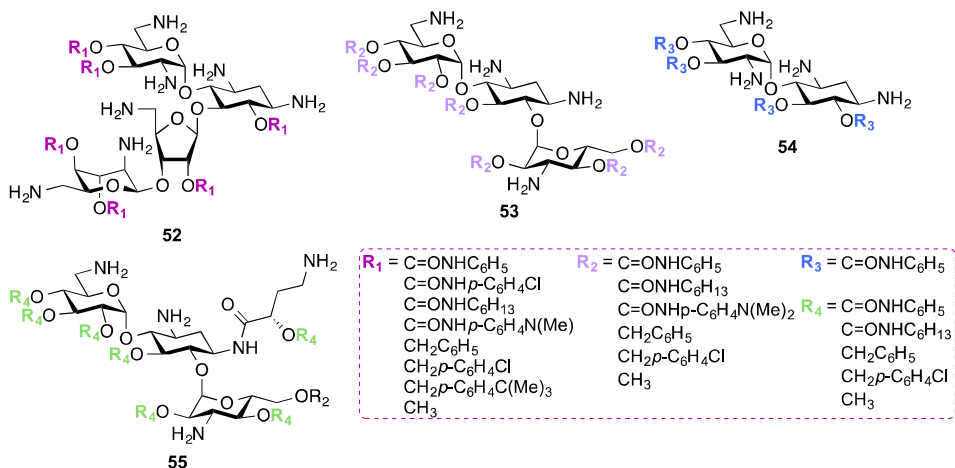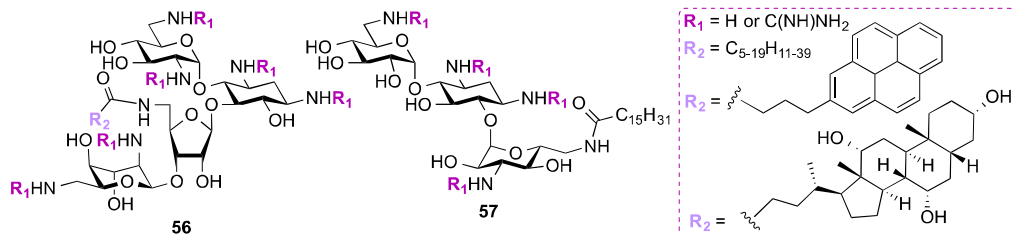
Fig. 6. Representative structures of **A.** NEA, RIB, and KANA conjugated to the pantetheinyl arm of CoA at their 6'-position, **B.** NEO- and KANA-CIP hybrids, **C.** NEO-, KANA-, AMK-, and NEA-lipid conjugates, and **D.** NEO- and KANA-polycationic lipid conjugates.

(Fig. 6B, **50-51**). The efficacy of these conjugates was tested against various strains of *E. coli*, *Bacillus subtilis*, and MRSA. The NEO-CIP hybrids were found to be 2 to 253-fold and 8 to 128-fold better than NEO against *E. coli* and MRSA, respectively. Similarly, notable antibacterial

activity was observed in the case of KANA derivatives in comparison to the parent AG. MIC values were determined against *E. coli* strains expressing APH(3') and the bifunctional AAC(6')-Ie/APH(2")-Ia enzymes, and the conjugates (6-48 μg/mL in the case of AAC(6')-Ie/APH(2")-Ia and 3-96 μg/mL in the case of APH(3')) were found to be less effective than CIP (0.38 and 0.10 μg/mL), but significantly better than NEO (>384 and 96 μg/mL). The emergence of bacterial resistance was tested by using CIP, NEO, a 1:1/CIP:NEO mixture, and the AG-CIP conjugates against *E. coli* and *B. subtilis* strains. The relative MIC values of CIP, NEO, and the 1:1/CIP:NEO mixture were increased by 75, 4, and 20-fold against *E. coli* and by 37.5, 8, and 7.6-fold against *B. subtilis*, whereas the conjugates remained unchanged in both cases.

Patent WO 2011/124986 A2[119]

In patent WO 2011/124986 A2,[119] a new class of AGs conjugated to lipids through their hydroxyl groups was synthesized (Fig. 6C, **52-55**). Oligocationic antibacterials such as AGs, which contain multiple positively-charged amine functional moieties, as well as peptides, lipopeptides, and their synthetic mimics are known to display a broad spectrum of activity.[120-124] In order to bind intracellular targets such as RNA, DNA, and proteins, the non-amphiphilic AGs have to cross the bacterial membrane. Therefore, co-administration of AGs and membrane-permeabilizing agents (*e.g.*, ionic lipids) can result in synergistic enhancement of antibacterial activity.[125, 126] In patent WO 2011/124986 A2, twenty different moieties of four different oligocationic AG-based polyol (NEO, KANA, AMK, NEA) were synthesized by converting the parent AGs to their polycarbamates and polyethers derivatives. The nature of the polyol scaffold, number of positive charges, and hydrophobic groups had a significant impact on their antibacterial activity. The NEO-based hexacationic heptaphenylcarbamate (**52** with R_1 = C=ONHC$_6$H$_5$) was found to exhibit antibacterial activity against Gram-positive MRSE, *S. aureus*, MRSA, and *S. epidermidis* (MIC ≤1 μg/mL). A remarkable 256-fold enhancement in antibacterial activity was observed for NEO-based hexacationic heptaphenylcarbamate against MRSA when compared to NEO while maintaining potent activity against NEO-susceptible strains, such as MRSE, *S. aureus*, *S. epidermidis,* and *K. pneumoniae*. In contrast, the tetracationic KANA, AMK, and NEA-based polyphenylcarbamates exhibited up to 64-fold reduced Gram-positive activity. Replacement of the polyphenylcarbamate by polybenzylether also resulted in potent antibacterial AGs. The most potent hexacationic NEO-based heptabenzylether (**52** with R_1 = CH$_2$C$_6$H$_5$) displayed slightly decreased Gram-positive activity (MIC = 2-4 μg/mL) against *S. aureus*, MRSA, *S. epidermidis*, and MRSE in comparison to its polycarbamate counterpart. In the case of both polycarbamates and polyethers, *para*-substitution on the aromatic ring of NEO, KANA, and AMK resulted in a 4 to 64-fold decrease in antibacterial activity. The effects of substitution pattern on the aromatic ring on antibacterial activity suggest contribution of factors other than polycationic charges and amphiphilicity on their mechanism(s) of action.[127]

Patent US 2013/0053337 A1[128]

In addition to the AG-lipid conjugates presented in patent WO 2011/124986 A2,[119] KANA- and NEO-lipid conjugates, with free or guanidinylated amines are presented in patent US 2013/053337 A1[128] (Fig. 6D, **56-57**).[129, 130] Fourteen NEO- and two KANA-based polycationic lipid hybrids were synthesized. A variety of lipophilic moieties (*e.g.*, C$_6$, C$_{12}$, C$_{16}$, C$_{20}$, doubly unsaturated C$_{18}$, pyrene, and cholic acid) were selected to investigate the effects of the hydrophobic tails on the antibacterial activity of these AGs. The antibacterial activity of these hybrids was tested against both Gram-positive and Gram-negative bacterial strains, including *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis*, *E. faecium*, *E. coli*, and *P. aeruginosa*. Overall, it was found that, in order to be active, the lipid chains of these hybrids needed to comprise at least 12 carbons, and 16 carbons was shown to be optimal. It was also observed that, when derivatized with the same lipid chain, the NEO scaffold was always more active than its KANA counterpart. Finally, the guanidinylated hybrids were found to be more active than their non-modified counterparts. Other examples of amphiphilc NEO-peptide derivatives have also been reported in the literature.[131-133]

Aminoglycosides as antifungal agents

As described in the section above, traditionally AGs are used for treating bacterial infections in humans and animals, but continuous setbacks associated with their antibacterial activity have prompted scientists to explore new targets for AGs. The lack of efficient antifungal agents, the increase in the number of immunocompromised patients, as well as the widespread resistance to existing antifungal agents have culminated in the emergence of fungal disease as a major public concern.[134-136] Common fungal infections like athlete's foot, ringworm, candidiasis, and serious systemic infections such as cryptococcal meningitis are treated by using FDA approved antifungal agents (*e.g.*, amphotericin B), but the toxicity levels of these antifungals are a common concern for medical practitioners.[137] Fungal and oomycete infections are responsible for plant diseases, and direct application of biocides to plant fungi is a common and efficient strategy used for their treatment. Unfortunately, inconsistent and declining efficacy, environmental impact, and toxicity have resulted in lower impact of these agents on plant fungal infections. An early report by Lee and co-workers on the activity of AGs against six crop pathogenic oomycetes (*Phytophthora* and *Pythium* species) and ten common fungi provided insight into the use of AGs as an alternative for treating fungal infections.[138]

The widely used antifungal agents are either sterol-binding polyene compounds (*e.g.*, amphotericin B) or sterol biosynthesis disrupting azoles (*e.g.*, fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR)), but toxicity and resistance associated with these molecules have created a shortage of effective antifungal agents.[139, 140] Thus, the development of newer antifungals based on AGs possessing novel mechanism(s) of action is currently underway. Generating novel amphiphilic AGs by attaching alkyl or other hydrophobic groups to parent AGs was found to alter the microbial properties of these compounds. Extensive efforts towards generating amphiphilic AGs by attachment of hydrophobic groups to AGs have recently been reported.[77, 78, 127, 141-148]

REVIEW

A. Patent US 2011/0130357 A1

B. Patent US 2014/025665 A1

58: R₁ = H, R₂ = C₄H₉
59: R₁ = H, R₂ = C₈H₁₇
60: R₁ = H, R₂ = C₁₂H₂₅
61: R₁ = H, R₂ = C₈H₁₇

62: R = C=OC₇H₁₅
63: R = C=OC₉H₁₉
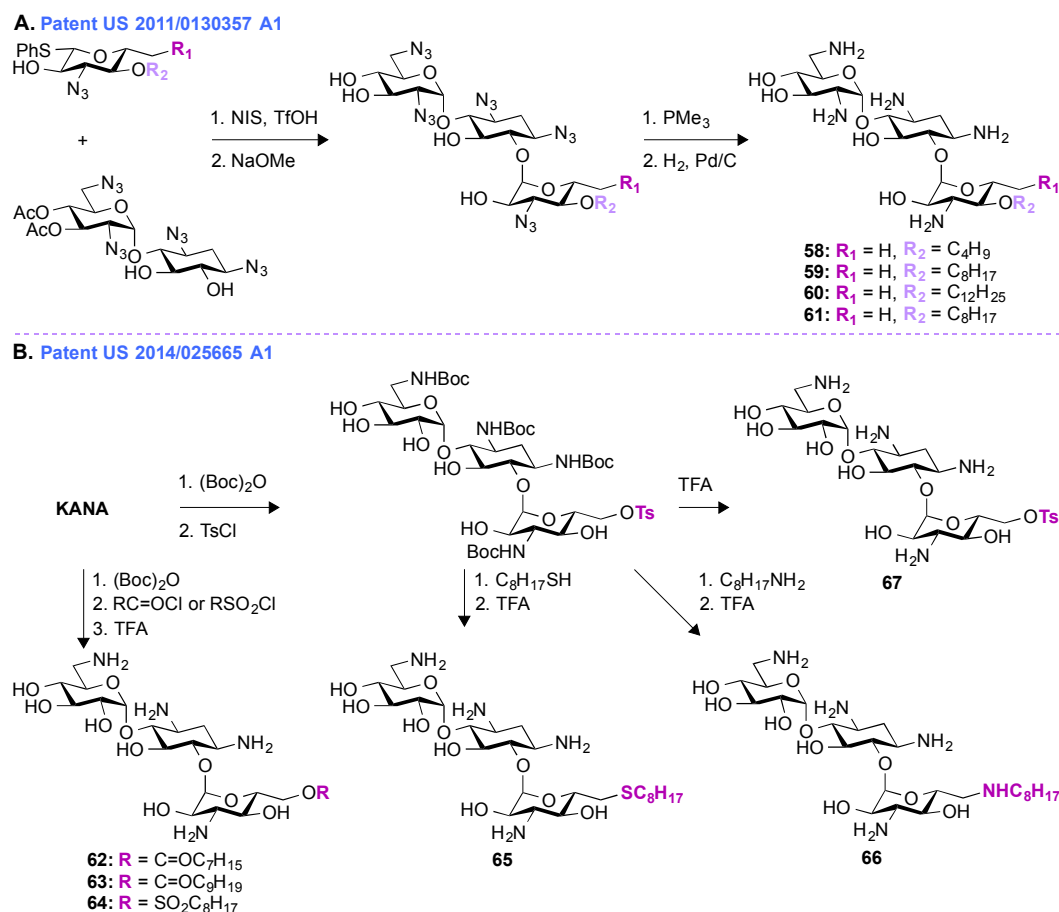**64:** R = SO₂C₈H₁₇

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66

67

Fig. 7. A. Representative scheme for the preparation of **A.** 6"-alkylated KANB amphiphiles, and **B.** 6"-alkylated KANA amphiphiles with ether, thioether, and amine linkages.

The length of the alkyl chain, the chemical nature of the hydrophobic
5 moiety, and the site of attachment on the AG scaffold significantly influence the behavior of these amphiphilic AGs towards microbial infections. The work from the Chang and Takemoto groups has highlighted the discovery of amphiphilic KANA and NEO analogues that are antifungal, but not antibacterial and they inhibit the fungal growth by
10 membrane perturbation.[149, 150]

The recent development of amphiphilic AGs with mechanisms of action, which differ from traditional AGs, can represent a significant advancement towards more effective AGs.[151] There are two common strategies for the synthetic production of amphiphilic AGs (i) direct
15 modification using AGs as starting materials and (ii) diversification of ring III using glycosylation.[152-158] The direct modification approach was employed for generating KANA and TOB analogues by using Boc chemistry to protect amines followed by nucleophilic substitution reaction at the only primary 6"-OH using various functional groups such as
20 thioether, alkoxyl, alkylamino, alkylamido, and esters. Modifications on ring III of KANB at the 3"-, 4"-, and 6"-positions yielded little to no antibacterial activity, but resulted in inhibition of various yeasts, oomycetes, and fungi with MIC values ranging from 3.9 µg/mL to 31.3

µg/mL, highlighting the significance of the alkyl chain in antifungal
25 switch.[149, 159] Recent work from Garneau-Tsodikova and co-workers has highlighted the *in vitro* antifungal activity, cytotoxicity, and membrane-disruptive action of amphiphilic KANB and TOB analogues bearing a thioether linkage at their 6"-position.[143, 160, 161] TOB analogues with C₁₂ and C₁₄ chains showed promising activities against fungal strains with
30 MIC values ranging from 1.95 to 62.5 mg/L and 1.95 to 7.8 mg/L, respectively.[161] In the case of KANB, analogues with C₁₂, C₁₄, and C₁₆ chains were 4, 16, and 32-times more effective than KANB against several of the yeast strains with MIC values ranging from 1.95 to 31.2 µg/mL.[160] TOB C₁₂ and C₁₄ analogues were tested for synergistic
35 combination effect against *C. albicans* strains with four azoles.[162] The combination effects of C₁₂ or C₁₄ and azoles appears non toxic to mammalian cells at higher or equal to synergistic antifungal MIC values of these drugs against fungi. This section of the review summarizes the recent application of AGs as antifungal agents exploring their synthesis
40 using direct modification and glycodiversification strategies.

Patent US 2011/0130357 A1[163]

In patent US 2011/0130357 A1, a glycodiversification strategy was used

to synthesize various analogues of KANA and KANB as antifungal agents (Fig. 7A, **58-61**). The present invention relates to novel analogues of AGs with substituents at the 6"-position of ring III which exhibit enhanced antifungal activity, but with minimum antibacterial property.

5 Modifications at the 3"-, 4"-, and 6"-positions of the antibacterial KANB resulted in the antifungal agent **59**. Compound **59** and its analogues were synthesized *via* successive regio- and stereoselective glycosylation of an azido-neamine core, deacetylation, Staudinger reduction of the azido groups, and hydrogenolysis of the benzyl groups. Different alkyl chains

10 ranging from C_4 to C_{12} in length were introduced at the O4-position of ring III and C_8 was found to be the optimum chain length for efficient antifungal activity. **59** and its derivatives were tested against various fungal strains and for **59**, the MIC value was found to be 31.3 μg/mL against *Fusarium graminearum*. To understand the specificity of

15 compound **59** for fungi, its mechanism of action was studied using yeast *Saccharomyces cerevisiae* and the result showed the influence of plasma membrane permeability for growth inhibition.[150] To further elucidate SARs for this phenomenon, KANB derivatives were synthesized using glycodiversification and the 3"-OH was found to be better than the 3"-

20 NH$_2$, and deoxygenation at the 6"-OH was found to be unnecessary for their activity.[159]

Patent US 2014/0256665 A1[164]

In patent US 2011/0130357 A1, the synthesis of compound **59** and its derivatives were carried out using glycodiversification reactions and, even

25 though these molecules showed considerable antifungal activity, the complex synthetic protocol previously patented was found to be not suitable for scale-up. Thus, based on the SAR study of **59**, a simplified method of direct modification of AG was developed in patent US 2014/0256665 A1[164] to produce another set of amphiphilic molecules

30 from tetra Boc-protected KANA (Fig. 7B, **62-67**). The synthesis followed direct introduction of esters and sulfones to the Boc-protected KANA as well as introduction of amines and thioethers using substitution reactions. These AGs were used in combination with azoles in different combinations ranging from 1:1 to 1:1000 (AG:azole). The most active

35 KANA derivative was found to be compound **64**. The relative growth inhibitory activities for **64** were tested against various bacterial and fungal strains and the MIC value for **64** against *F. graminarum* was found to be between 7.8 and 15.6 μg/mL. Compound **64** was tested against *E. coli* and *Micrococcus luteus* and its MIC values were found to be 250 and 62.5

40 μg/mL, respectively (*Note*: KANA MIC values = 0.98 and 1.95 μg/mL, respectively). These data demonstrated the inherent lower antibacterial activity and greater antifungal property of **64**. Synergistic inhibition assays were performed with **64** and a variety of azoles (ITC, FLC, POS, VOR, clotrimazole, tioconazole, ketoconazole, metconazole, and

45 pyraclostrobin) against an azole-resistant *Candida albicans* strain. These combinations showed synergistic effect with FICI values ranging from 0.13-0.31 μg/mL. Finally the synergistic effect was confirmed by time-kill curve study against that *Candida albicans* strain. To further understand the mechanism of action of compound **64**, fungal models were

50 used and it was found that the fungicide action results from membrane perturbation, which is influenced by lipid modulation.[165]

Newer applications of aminoglycosides

Apart from their use in treatment of microbial infections, research has recently focused on exploring AGs for potential treatment of genetic

55 disorders. The genetic disorders for which AGs are currently investigated result from nonsense mutations where a premature termination codon

(PTC) replaces an amino acid coding-codon to interfere with protein translation resulting in truncated and inactive proteins.[166-169] Base pair insertion, deletion, or substitution result in the formation of PTCs, which

60 ultimately result in a number of genetic disorders such as cystic fibrosis (CF),[170] Lafora disease, Duchenne muscular disorder (DMD),[171] Rett syndrome,[172] spinal muscular dystrophy,[173] congenital muscular dystrophy,[174] Type I Usher disease,[175] ataxia-telangiectasia,[176] Hurler syndrome,[177] hemophelia A, hemophelia B,[178] and Tay-sachs. At the

65 present, there are no effective treatments available for these disorders. Even though gene therapy has been considered a potential solution for these disorders, there are still many critical problems to be solved before using this technique in humans. AGs act against microbial infections by binding to A-site of the 16S rRNA and this mechanism of action has been

70 exploited for their ability to readthrough stop codon mutations, generating full-length proteins from part of the mRNA molecules.[179-182] Studies have shown several parent AGs, such as AMK and GEN, to promote readthrough of the PTC in mouse models and patient samples.[183-185] Additionally, derivatives of PAR such as NB30, NB54, NB74, NB84, and

75 NB124 were reported to suppress PTCs and display lower toxicity when compared to their parent AGs (Fig. 8).[186-191] Thus, research on AGs and their derivatives' ability to suppress PTCs while improving their potency and decreasing their toxicity is a promising direction to pursue for the discovery of treatment of the resulting disorders.
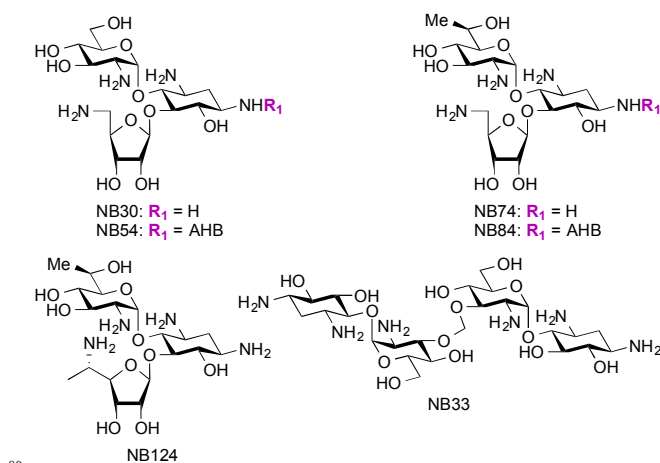

Fig. 8. Structures of NB derivatives discussed in the section "newer applications of AGs".

In addition to treatment of genetic disorders resulting from PTCs, AGs have been explored as potential anti-HIV (Human Immunodeficiency

85 Virus) agents. Research has shown that AGs and AG conjugates are capable of interfering with many steps of the HIV life cycle, thus providing a new application for AG-based compounds.[192, 193] The AGs conjugates NEO-Hoechst 33258 have been found to have higher affinity and specificity to HIV-1 trans-activation response element (TAR)

90 RNA.[194] AG-arginine conjugates (AArCs) and AG-polyarginine conjugates (APArCs) have also been extensively investigated as potential treatment of HIV.[195, 196] NEO and APR have also been found to bind HIV-1 dimerization initiation (DIS), which is responsible for genome initiation.[197, 198] Homo- and hetero-dimers of KANA, NEO, and TOB, as

95 well as naphthalene-based diimide-conjugated AGs were reported to bind to HIV-1 Rev response element (RRE).[199, 200] NEA dimers as well as nucleobase-AG conjugates were found to inhibit TAR-Tat binding[201] and guanidine derivatives of NEO were shown to bind the RNA helix of HIV-1 frameshift.[202] Overall, these studies demonstrate the additional novel

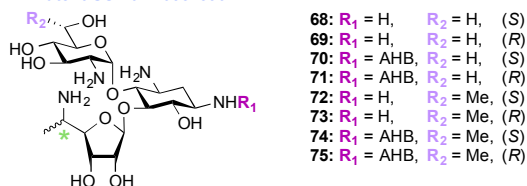uses of AGs, which will be the focus of this section.

Patent US 2014/0357590 A1[203]

In the treatment of genetic disorders associated with PTCs, the mechanism of action by which AGs bind to the 16S rRNA is used for enhancement of termination suppression by stop codons. AGs have been shown to suppress various stop codons with notably different efficiencies (UGA>UAG>UAA).[204] The first genetic disease examined was CF, and the experiments demonstrated AG-mediated suppression of CF transmembrane conductance regulator (CFTR) mutations especially by the members of the GEN family and G418.[170, 205] Although G418 displayed the best termination suppression activity in *in-vitro* experiments, its toxicity, even at low concentrations, was a major drawback. In patent US 2014/0357590 A1,[203] PAR-derived pseudotrisaccharide AGs were designed to replace GEN, which exhibited high premature stop-codon mutation readthrough activity while exerting low cytotoxicity and antimicrobial activity (Fig. 9A, **68-75**). Based on the results from patent WO 2007/113841,[206] where the derivative NB30 exhibited reduced cytotoxicity compared to PAR and GEN while promoting dose-dependent suppression of nonsense mutation of the PCDH15 gene in the case of Usher's disease but its suppression potency was lower than PAR and GEN,[175, 207] NB54 was developed as a second-generation concept structure, which exhibited substantially higher readthrough efficiency than PAR and GEN. Furthermore, NB30 and NB54 were evaluated for readthrough efficiency for treatment of *USH1C* nonsense mutation, and *in vivo* administration of NB54 induced the recovery of full-length harmonin 1a with ehhanced efficiency.[208] NB54 was tested for its ototoxicity and enhanced suppression of CF PTCs. NB54 was shown to partially restore halide efflux in a CF bronchial epithelial cell line, but not in a CF cell line lacking a PTC.[186] In related research efforts, two novel analogues, NB30 and NB33, which are derivatives of G418 were synthesized and were found to display increased selectivity towards eukaryotic cells over prokaryotic cells. Cell toxicity tests using three kidney cell lines confirmed that NB30 is 6-15 fold less toxic than GEN and PAR. NB30 was shown to produce full-length proteins more efficiently than PAR or GEN. To address the enhanced affinity for eukaryotic cells, a comparative biochemical and structural analysis of NB30, NB33, together with a series of different 4-, 4,5-, and 4,6-substituted 2-DOS AGs in both eukaryotic and prokaryotic systems was performed. From these studies, it was revealed that NB33 is the strongest inhibitor of eukaryotic protein translation and it was found to preferentially bind the eukaryotic RNA decoding-site.[191, 209, 210]

In patent US 2014/0357590 A1, newer analogues **68-75** were synthesized by modifying ring I, ring II, and ring III of the pseudotrisaccharide (Fig. 9A). For the readthrough assays, the prevalent nonsense mutations chosen were R3X and R245X for USH1, G542X and W1282X for CF, Q70X for HS, and R3381X for DMD. For all the mutations tested, the efficacy for AG-induced readthrough was **70** > **71** > NB54 > GEN, suggesting better cell permeability for **70** and **71** over NB54 due to the presence of a 5"-methyl group. A similar trend was observed for another NB derivative, compound **74**, where two methyl groups ((*R*)-6'-methyl group (*S*)-5"-methyl groups) were shown to operate synergistically to enhance readthrough activity. The derivatives **68-75** were investigated as antibacterial agents against both Gram-negative and Gram-positive bacterial strains (*E. coli*, *B. subtilis*) and were found to have lower specificity for prokaryotic ribosome (IC$_{50}$ values ranging from 160 ± 20 to 2266 ± 196 nM) compared to GEN (28 ± 4 nM) and PAR (51 ± 5 nM), explaining their lower antibacterial activity. Cell toxicity data

demonstrated that **68** and **70**, which were formed by installation of a (*S*)-5"-methyl group on NB30 and NB54, respectively, did not significantly affect their cytotoxicity values (LC$_{50}$ values 21.4 and 23.5 ▢M for NB30 and **68**, respectively, and 6.1 and 10.1 ▢M for NB54 and **70**, respectively) whereas 1.9-fold and 4.6-fold increase in their stop codon suppression activity was observed. The ratios for prokaryotic *versus* eukaryotic selectivity found was found to be 2214 for GEN, 1118 for PAR, and 225 for G418, whereas compounds **68-75** displayed values between 0.4-151, which explained their lower cytotoxicity values.

A. Patent US 2014/0357590 A1

68: R$_1$ = H,   R$_2$ = H,   (*S*)
69: R$_1$ = H,   R$_2$ = H,   (*R*)
70: R$_1$ = AHB,   R$_2$ = H,   (*S*)
71: R$_1$ = AHB,   R$_2$ = H,   (*R*)
72: R$_1$ = H,   R$_2$ = Me,   (*S*)
73: R$_1$ = H,   R$_2$ = Me,   (*R*)
74: R$_1$ = AHB,   R$_2$ = Me,   (*S*)
75: R$_1$ = AHB,   R$_2$ = Me,   (*R*)

B. Patent US 2011/0046982 A1

76: Linker = Triazole, urea, thiourea-based
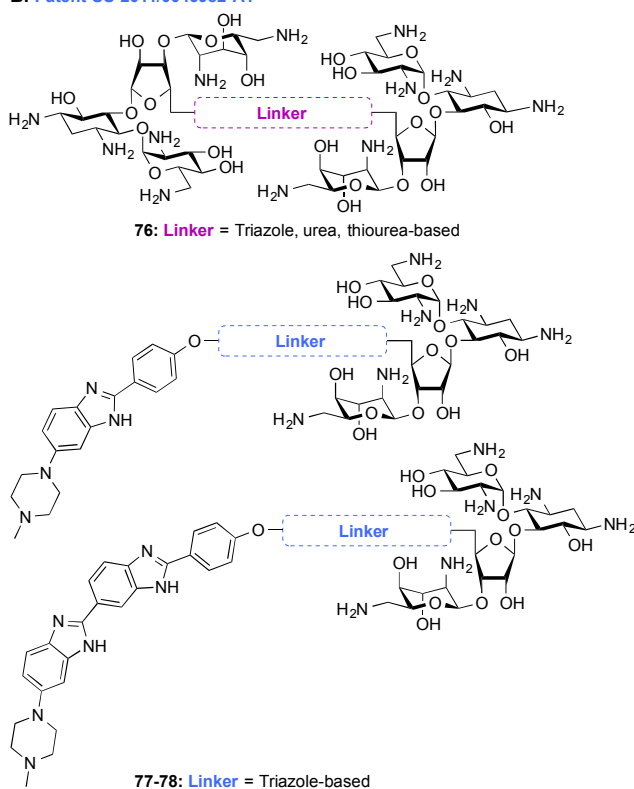
77-78: Linker = Triazole-based

Fig. 9. Representative structures of **A.** pseudotrisaccharide NB derivatives, and **B.** NEO dimers as well as NEO-benzimidazole and NEO-bisbenzimidazole conjuagtes with various linkers.

Patent US 2011/0046982 A1[211]

Acquired Immunodeficiency Syndrome (AIDS), which is caused by the HIV, is a global health issue and there is a great necessity for new therapies to treat this disease. In patent US 2011/0046982 A1,[211] AG-small molecule combinations were evaluated for their binding affinity and specificity to viral RNA, such as HIV TAR RNA. As NEO is able to inhibit Tat peptide binding to the TAR RNA and studies on the interactions of NEO with TAR helped identify three binding sites,[212, 213] in this patent, NEO dimers were investigated to enhance TAR specificity (Fig. 9B, **76**). Various aspects of different AG-small molecule conjugates in terms of their method of synthesis, efficiency for binding DNA, RNA,

and correlation of efficacy of these molecules with respect to the linkers used were explored in details.[194, 214-222] Additionally, as the benzimidazole Hoechst 33258 is capable inhibiting TAR region of HIV RNA, NEO-benzimidazole (analogues of Hoechst 33258) conjugates were also investigated (Fig. 9B, **77-78**). Conjugation of the AG NEO and benzimidazoles tethered by different alkyl linkers displayed high affinity binding to TAR with nanomolar IC_{50} values. UV melting studies were performed in the presence and absence of various ligands, NEO-benzimidazole derivatives **77-78** (4.4×10^7 M^{-1}) were found to have a higher binding constant than NEO (1.7×10^5 M^{-1}), Hoechst 33258 (4.2×10^5 M^{-1}), and benzimidazoles (5.2×10^5 M^{-1}) alone. The length of the linker affected the change in melting temperature and NEO-benzimidazole conjugate with 24 backbone carbon atoms showed the biggest change in melting temperature. Similarly, in the case of NEO dimers, the length of the linkers appeared to display a direct correlation with their change in melting temperature. These NEO conjugates provided maximum protection from HIV cytopathic effects (2-44%) at a varied range of concentrations (2-206 μM). These compounds were also tested for their efficiency to control the release of reverse transcriptase (RT), even though the cells were positive for HIV after 6 days of experiment they were shown to inhibit the RT release up to a limit. Finally, all these NEO derivatives were used to study competition-binding affinity against HIV-1 TAR RNA. The IC_{50} values for these compounds ranged from micro to nanomolar concentrations and their binding free energy were calculated. The dimers with less flexible linker were found to have better binding values.

Perspective and Conclusions

In the last decade, the field of AG antibiotics has experienced a renewed interest among the scientific community and efforts were made to counter the resistance and toxicity associated with these drugs. In that ten-year period, more traditional reviews on AGs have been published in the literature.[1, 6, 8, 36, 37, 151, 223-232] In this review, by summarizing all recent patents on AGs, we highlighted their use/development as antibacterials and antifungals, as well as those for potential newer applications (genetic disorders and HIV).

Even though extensive work has been done in the field of AGs, there are still many avenues left to explore. In recent years, a thorough study of the mechanism of action of AGs on bacterial ribosomes led to the discovery of the H69 binding site, a new target now available for further exploration. Several approaches have also been explored to counter the resistance caused by AMEs, including designing AME inhibitors, synthesizing AG scaffolds capable of evading the action of AMEs, and regulating AME expression.[37, 223-225] Even though metal cations have lately been shown to inhibit AAC activity and increase AG efficacy in AG-resistant bacterial strains,[226, 227] more detailed mechanistic investigations remain to be performed. The discovery of the lack of ototoxicity of APR also offers multiple prospects for the development of this relatively unexplored scaffold. The fields of AGs as antifungal agents and as treatments for PTC disorders are still in their infancy and surely provide a lot of opportunities for investigation. These areas of study will probably be some of the most exciting for AG developers to pursue in the future. In conclusion, the area of research on AGs has recently experienced a renaissance and promise to remain for a long time a valuable source for drug discovery and development.

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Notes and references

^a *University of Kentucky, Department of Pharmaceutical Sciences, 789 South Limestone Street, Lexington, KY, USA. Fax: 859-257-7585; Tel: 859-218-1686; E-mail: sylviegtsodikova@uky.edu*

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