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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.9b12420 • Publication Date (Web): 20 Jan 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Chemical Modifications Reduce Auditory Cell Damage Induced by Aminoglycoside Antibiotics

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ABSTRACT: Although aminoglycoside antibiotics are effective against Gram-negative infections, these drugs often cause irreversible hearing damage. Binding to the decoding site of eukaryotic ribosomes appears to result in ototoxicity, but there is evidence that other effects are involved. Here we show how chemical modifications of apramycin and geneticin, considered amongst the least and most toxic aminoglycosides, respectively, reduce auditory cell damage. Using molecular dynamics simulations, we studied how modified aminoglycosides influence the essential freedom of movement of the decoding site of the ribosome, the region targeted by aminoglycosides. By determining the ratio of a protein translated in mitochondria to that of a protein translated in the cytoplasm, we showed that aminoglycosides can paradoxically elevate rather than reduce protein levels. We showed that certain aminoglycosides induce rapid plasma membrane permeabilization and that this non-ribosomal effect can also be reduced through chemical modifications. The results presented suggest a new paradigm for the development of safer aminoglycoside antibiotics.

INTRODUCTION

For almost eight decades, aminoglycosides (AGs) have been clinically useful antibiotics.[1] First discovered in 1943, the AG streptomycin is still used today in treatment of tuberculosis including cases of infection with multidrug-resistant *Mycobacterium tuberculosis*.[2–4] AGs are key drugs for treatment of neonatal sepsis[5], a potentially fatal infection. Cystic fibrosis patients often suffer from reoccurring lung infections, and AGs markedly slow the decline in lung function in these patients.[6]

Unfortunately, the efficacy of AGs is greatly overshadowed by nephrotoxicity, which is largely reversible, and irreversible ototoxicity caused by these antibiotics. AGs are poorly metabolized drugs and reach the kidneys largely intact, thereby retaining both their desired antibacterial activity and undesired adverse effects.[7] Treatment with AGs can, therefore, result in acute kidney injury and increase the risk of developing a chronic kidney disease. In the kidney, AGs accumulate in proximal tubular cells where they disrupt several intracellular processes, leading to tubular epithelial cell death and tubular necrosis.[8]

AGs may also cause ototoxicity, which is inner ear dysfunction with symptoms of hearing loss and/or dizziness. Some AGs are more toxic to the cochlea, the inner ear part responsible for hearing, whereas others are more toxic to the vestibular apparatus, responsible for balance.[9,10] Ototoxicity is dose dependent, and certain patients are genetically more susceptible than others.[11–15] The severity of AG-induced ototoxicity was analyzed in a study of 153 adult cystic fibrosis patients who were treated with AGs: About 42% suffered mild ototoxic side effects, and about 9% had ototoxicity that ranged from moderate to severe.[16] In cystic fibrosis patients, life-threatening infections and lack of suitable alternative antibiotics often make it necessary to continue treatment with AG antibiotics despite their toxicity.

Although the effects of AGs on the kidney proximal tubular epithelial cells and inner ear sensory cells have been extensively studied, whether the exact same molecular mechanisms lead to ototoxic and nephrotoxic effects is unclear.[17,18] There has been some success in development of AGs that are less prone to modifications that confer resistance to these antibiotics, yet no AGs specifically developed to reduce toxicity are available for clinical use so far.[19–22]

AGs interfere with bacterial protein synthesis by binding to the A-site of the bacterial ribosome; the A-site binds tRNA during the mRNA decoding process.[23] Crystallographic studies of complexes between different AGs and the bacterial A-site suggest that these antibiotics stabilize an A-site conformation similar to that induced by the binding of cognate tRNA to the bacterial 30S ribosome subunit.[24,25] Studies published over the years suggest that perturbation of mammalian cell translation is responsible for the toxicity of AG antibiotics: The relatively high sequence similarity between the decoding A-site of bacterial ribosomes and mammalian mitochondrial and cytoplasmic ribosomes leads to limited selectivity, and non-specific ionic interactions between the highly positively charged AGs and negatively charged rRNA backbone further limit the selectivity of these antibiotics.[26–29] Clear evidence for the effects of AGs on mitochondrial translation were obtained by the Böttger group through isolation of mitochondria from mammalian cells pretreated with an AG and by development of *in vitro* translation assays using hybrid ribosomes engineered to have the A-site sequence of the human mitochondrial ribosome in an otherwise bacterial ribosome context.[30,31]

Using an *in vitro* model, Francis and co-workers showed that AGs' toxicity can also be correlated with the extent of inhibition of cytoplasmic translation.[27] In addition, several clinically used AGs, including tobramycin and gentamicin, display similar potencies as inhibitors of mitochondrial and cytosolic eukaryotic translation in cell-free systems.[32] These data suggest that perturbation of the fidelity of both mammalian translation machineries contribute to the toxicity of AG antibiotics.

In search of strategies to selectively enhance inhibition of bacterial translation by AGs we previously reported that modifying the C-5 alcohol of certain AGs composed of a 4,6-disubstituted 2-deoxystreptamine with a β-O-linked ribofuranose can improve selectivity for the bacterial ribosome.[33] Crich and coworkers recently introduced propylamycin, a 4′-deoxy-4′-propyl semisynthetic derivative of paromomycin that has potent antibacterial activity. Using *in vitro* translation assays with hybrid ribosomes they showed that propylamycin has higher affinity for the bacterial ribosome compared to human mitochondrial or cytosolic ribosomes. Propylamycin is also less ototoxic than paromomycin in guinea pigs.[34]

The mechanisms of AG-induced ototoxicity appear to differ depending on the specific AG. An accumulation of experimental evidence indicates that different AGs not only have differences in binding to the A-site but also differ in off-target activities associated with their toxicity to mammalian cells in general and auditory cells in particular. Production of toxic concentrations of reactive oxygen species is a well-documented effect of AGs on mammalian cells that, in turn, triggers apoptosis.[35–37] However, this effect may be a consequence of perturbation of translation that leads to elevated levels of dysfunctional mitochondrial proteins, especially those involved in the respiratory chain complexes that facilitate mitochondrial electron transport.[38]

AGs are cationic under physiological conditions, and this allows these drugs to displace natural bivalent cations responsible for membrane integrity leading to cell permeabilization. Interactions between bivalent cations such as Ca^{2+} and Mg^{2+} and lipids facilitate the structural integrity and function of the plasma membrane.[39] It was previously shown that AGs like streptomycin and isepamicin markedly increase permeability of the mammalian cell membrane, whereas other such as gentamicin have little effect on mammalian cell membranes.[40] This indicates that both the cationic nature and the specific structure of an AG are related to off-target effects. The membrane-disrupting effects of AGs have been enhanced in the past decade by development of AG-based antimicrobial cationic amphiphiles that rapidly disrupt cell-membrane integrity.[41–46]

Depending on the AG and the administration regimen, the maximal plasma concentrations of AGs range from about 10 to about 100 μg/mL.[47–50] It remains an open question whether AGs reach the auditory hair cells at concentrations sufficient to perturb cytosolic and mitochondrial translation. It should also be noted that perturbation of mitochondrial translation requires that the AG cross the cytoplasmic membrane as well as both the outer and inner mitochondrial membranes.

To identify chemical modifications that improve the safety profile of AGs while preserving antibacterial efficacy, the relationships between specific structural motifs and toxicity must first be established. With the goal of identifying a molecular basis for the structure/ototoxicity relationship of AGs, we investigated how chemical modifications that do not significantly affect the antibacterial activity affect key functions of immortalized inner ear cells that express genes characteristic of inner-ear sensory hair cells.

RESULTS AND DISCUSSION

Ribosylation and N-demethylation of the AGs apramycin and geneticin. We focused our investigation on apramycin (APR) and geneticin (G-418) (Figure 1); these two AGs are at the two ends of the spectrum of AG-induced ototoxicity. APR induces only modest hair cell damage in cultures of cochlear explants as well as in several animal models including a guinea pig model of chronic ototoxicity.[51–53] G-418, on the other hand, is considered the most toxic AG and is not in clinical use.[28] Several previous reports provided evidence that APR inhibits translation but does not enhance misreading errors in bacterial and mammalian cytosolic ribosomes.[51,53–56] In contrast, investigations of G-418 bound to cytosolic A-site and to the bacterial A-site revealed that rather than inhibiting translation, this AG promotes missense errors during protein synthesis.[57,58] We examined the effects of two types of modifications to these AGs (Figure 1): 5-O-ribosylation (compounds **1**, **3**, **4**, **7**, and **9**) and N-demethylation of the secondary amine (compounds **2** and **5**). To evaluate the significance of ribosylation of the complete AG scaffold, this modification was carried out on the truncated pseudo-

disaccharide scaffolds of APR and G-418 (compounds **6** and **8**) to afford the corresponding ribosylated derivatives (**7** and **9**).

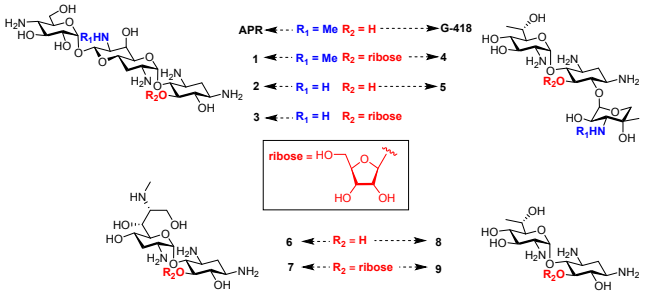

Figure 1: Structures of the AGs APR and G-418 and their semisynthetic derivatives.

Briefly, N-demethylation products of APR and G-418 were readily formed as separable products of an azido-transfer reaction to protect the primary amines of the parent AGs.[59] The formation of N-demethylated APR under the conditions of the azido-transfer reaction was previously reported by Crich and coworkers; an improved and selective protocol for de-methylation of APR using iodine under basic conditions was also reported by the same group.[60] Selective acetylation of all alcohols of the resultant azido-protected AGs with the exception of the desired 5-OH yielded AG-derived glycosyl acceptors; these were glycosylated with the 2,3,5-tri-O-benzoyl-D-ribofuranosyl-trichloroacetimidate followed by two to three deprotection steps to afford the ribosylated derivatives. Detailed synthetic schemes and procedures and compound characterization data are described in section 1 in the supporting information.

Ribosylation and N-demethylation have opposite effects on antibacterial activities of APR and G-418. The antibacterial activities of the parental and semisynthetic AGs were evaluated on a panel of Gram-negative and Gram-positive bacterial pathogens. Minimal inhibitory concentration (MIC) values were determined using the broth double-dilution method. Results of the antibacterial activity tests are summarized in Figure 2 and Supporting Table S1. The MIC values of compound **1**, the ribosylated derivative of APR, were comparable or differed by no more than 4-fold from those of the parent AG (Figure 2). These results are in agreement with those of Abe and coworkers who reported an alternative synthesis of compound **1** in 1981.[61] In contrast, and in agreement with previous observations made by Crich and coworkers, the MIC values of **2**, the N-demethylated derivative of APR, were higher than those of APR by 2 to 8 fold.[60] Interestingly, ribosylation of **2** resulted in enhancement of antibacterial activity; the MIC values of ribosylated derivative **3** were either comparable or 2-fold improved than those of **2** (Figure 2).

In contrast to its effect on the APR scaffold, ribosylation of G-418 led to a significant drop in antibacterial activity. Compared to G-418, the MIC values of **4**, the ribosylated derivative of G-418, were higher by at least 8 fold against the majority of the tested panel (Figure 2). N-demethylation of G-418 had little to no effect on its antibacterial activity. MIC values of **5**, the N-demethylated derivative of G-418, were the same or differed by no more than 2 fold from those of the parent AG against most of the strains tested.

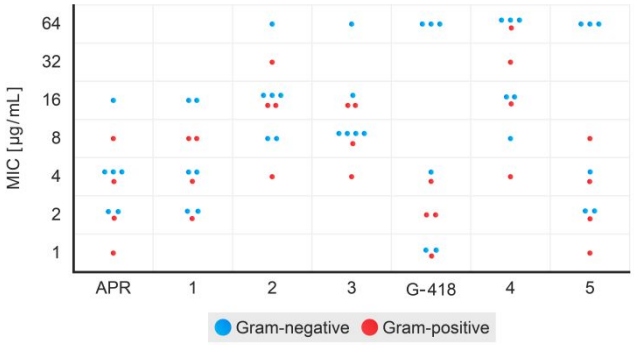

Figure 2. Minimal inhibitory concentrations of APR and G-418 and their semisynthetic derivatives. Plots of MIC values for indicated compounds. Blue dots represent Gram-negative strains and red represent Gram-positive strains. MIC values were determined using the broth double-dilution method. Each concentration was tested in triplicate, and results were reproduced in two independent experiments.

Ribosylation of C-5 alcohol of the pseudo-disaccharides neamine and nebramine yields the natural AG ribostamycin and its semi-synthetic derivative 3'-deoxy-ribostamycin. Both have markedly improved antibacterial activity compared to the corresponding pseudo-disaccharides from which they are derived.[33] However, with MIC values of 64 μg/mL or higher against most of the tested strains, APR-derived pseudo-disaccharide **6** and its 5-*O*-ribosylated derivative **7** were poor antibacterial agents (Supporting Table S1). Similarly, G-418 derived pseudo-disaccharide **8** and its ribosylated derivative **9** were not toxic to bacterial cells. These results indicate that 5-*O*-ribosylation cannot be applied as a general strategy to restore or improve the antibacterial activity of pseudo-disaccharide segments generated from AGs belonging to the 4,6-disubstituted series.

In summary, N-demethylation and ribosylation had opposite effects on APR and G-418. Whereas the APR scaffold tolerated 5-O-ribosylation, N-demethylation reduced its antibacterial efficacy. The reverse trend was observed for G-418. Ribosylation of G-418 abrogated its antibacterial activity, and N-demethylation of this AG had little or no effect on its antibacterial activity.

Effects of ribosylation and N-demethylation on the selectivity of inhibition of bacterial translation *in vitro*. To determine how ribosylation and N-demethylation affect bacterial and eukaryotic cytosolic translation, we focused on APR and its derivatives **1** and **3** and on G-418 and its derivative **5**; these were the most potent antibacterials of the AGs tested. We measured effects of these compounds on translation in cell-free extracts containing ribosomes isolated from *E. coli* or from the cytosol of rabbit reticulocytes. The concentrations at which the tested compounds inhibited luciferase activity

by 50% (IC_{50}) were determined, and the results are summarized in Figure 3. The IC_{50} values measured for APR are in good agreement with those previously reported by Böttger and coworkers.[60] Selectivity for bacterial translation was calculated as the ratio of the IC_{50} value for cytosolic translation divided by the IC_{50} value for bacterial translation, and the results are summarized in Figure 3.

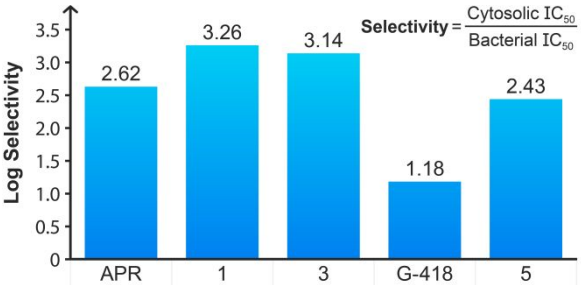

Figure 3. Selectivity of APR, G-418, and compounds 1, 3, and 5 for inhibition of cytosolic translation vs. bacterial translation. Selectivity values, calculated as the cytosolic IC_{50} divided by the bacterial IC_{50} are plotted on a $\log_{10}$ scale with the value above the bar. The IC_{50} values for the *in vitro* inhibition of cytosolic and bacterial translation and their standard deviations are given with units in μM below the plot. Each concentration was tested in duplicate, and the results are the averages of two independent experiments.

Ribosylation of APR enhanced selectivity to *E. coli* ribosomes; the selectivity of the ribosylated derivative **1** was higher than that of APR (Figure 3). APR and its ribosylated derivative **1** were recently evaluated by Böttger, Crich, and coworkers as inhibitors of engineered bacterial ribosomes in which the native A-site was replaced by a 30-basepair sequence of the human mitochondrial A-site.[62] Similar to the trend observed for inhibition of mammalian cytosolic translation, ribosylation of APR decreased the undesired inhibition of the hybrid ribosomes that contained the mitochondrial A-site by approximately 4-fold. This indicates that ribosylation of APR reduces its undesired inhibition of both mitochondrial and cytosolic mammalian translation.

N-demethylation considerably improved the selectivity of G-418 to inhibition of *E. coli* ribosomes. Compared to the parent AG, the selectivity of compound **5** increased by over an order of magnitude. Of note, the potencies of G-418 and **5** as inhibitors of bacterial translation were similar (Figure 3); the higher selectivity of **5** resulted from a considerably lower inhibition potency in the mammalian cytosolic translation system. This result is of particular interest since N-demethylation of G-418 reduced the undesired inhibition of cytosolic eukaryotic translation without significantly affecting its antibacterial activity.

The impact of chemical modifications on the flexibility of bacterial and human A-sites. During the bacterial translation process, A-site ribonucleotides A1492 and A1493 adopt different conformations. In the "on" state, the two adenines are bulged out from the A-site helix, and they participate in the stabilization of the Watson-Crick base pairs between the codon of the mRNA and the anticodon of the tRNA.[24,25,63] In the "off" state, the two adenines are stacked in the A-site double helix. Interactions between the AG and the A-site nucleotides stabilize the on-state.

To prevent decoding errors, the A-site must maintain a conformational balance between on and off states. Upon binding to an AG, the A-site rigidifies, and the freedom of movement required to achieve optimal speed and accuracy of translation is compromised. The movement of the A-site in the presence and absence of AGs was explored by Pilch and coworkers using a time-resolved fluorescence anisotropy assay.[64] Their results suggest that in designing AGs intermolecular and intramolecular dynamics should be addressed. Blanchard and coworkers investigated the structural and dynamic impacts of 4,5-linked AG antibiotics on the *E. coli* 70S ribosome via a single molecule FRET system.[65] By harnessing *in silico* molecular dynamics (MD) simulations Trylska and coworkers studied the effects of AGs on ribosome function and dynamics.[66]

Here we used MD simulations to study how the derivatives of APR and G-418 affect the flexibility of the bacterial and eukaryotic A-sites. Binding of all AGs to the A-sites occurs in the vicinity of adenines A1492 and A1493 in bacteria, cytosolic, and mitochondrial A-sites.[24,57,67,68] For docking calculations and MD simulations, we used fragments consisting of the 24 ribonucleotides surrounding these adenines in each site (Figure 4A-C). For the bacterial A-site structure, we cropped a sequence of 24 ribonucleotides from a complex of the bacterial 30S ribosome subunit with APR (PDB code: 4AQY).[51]

For simulations of the cytosolic A-site two structures were used. The first was from the X-ray structure of a complex between an oligo ribonucleotide mimic of the cytosolic A-site and APR (PDB code: 2G5K).[55] To facilitate crystallization, this RNA sequence contained several ribonucleotides that differ from those in the conserved *Homo sapiens* cytosolic A-site sequence. For MD simulations, these ribonucleotides were replaced with the conserved residues (Supporting Figure S1). The second structure was of the yeast 80S ribosome in complex with G-418 (PDB code: 4U4O)[69]; the A-site sequence of this rRNA is identical to that of the cytosolic *H. sapiens* A-site.

Since there are currently no available X-ray structures of complexes of the mitochondrial A-site with APR or G-418, the 24-nucleotide sequence of the mitochondrial apo A-site rRNA was cropped from the cryogenic electron microscopy structure of the mammalian mitochondrial ribosome (PDB code: 5AJ3). In this structure, A1492 and A1493 are bulged out of the helix.[29]

APR and G-418 were first docked into the selected structures using rDock, which successfully reproduced the binding modes of APR and G-418 observed in the crystal structures of bacterial and human cytosolic A-sites.

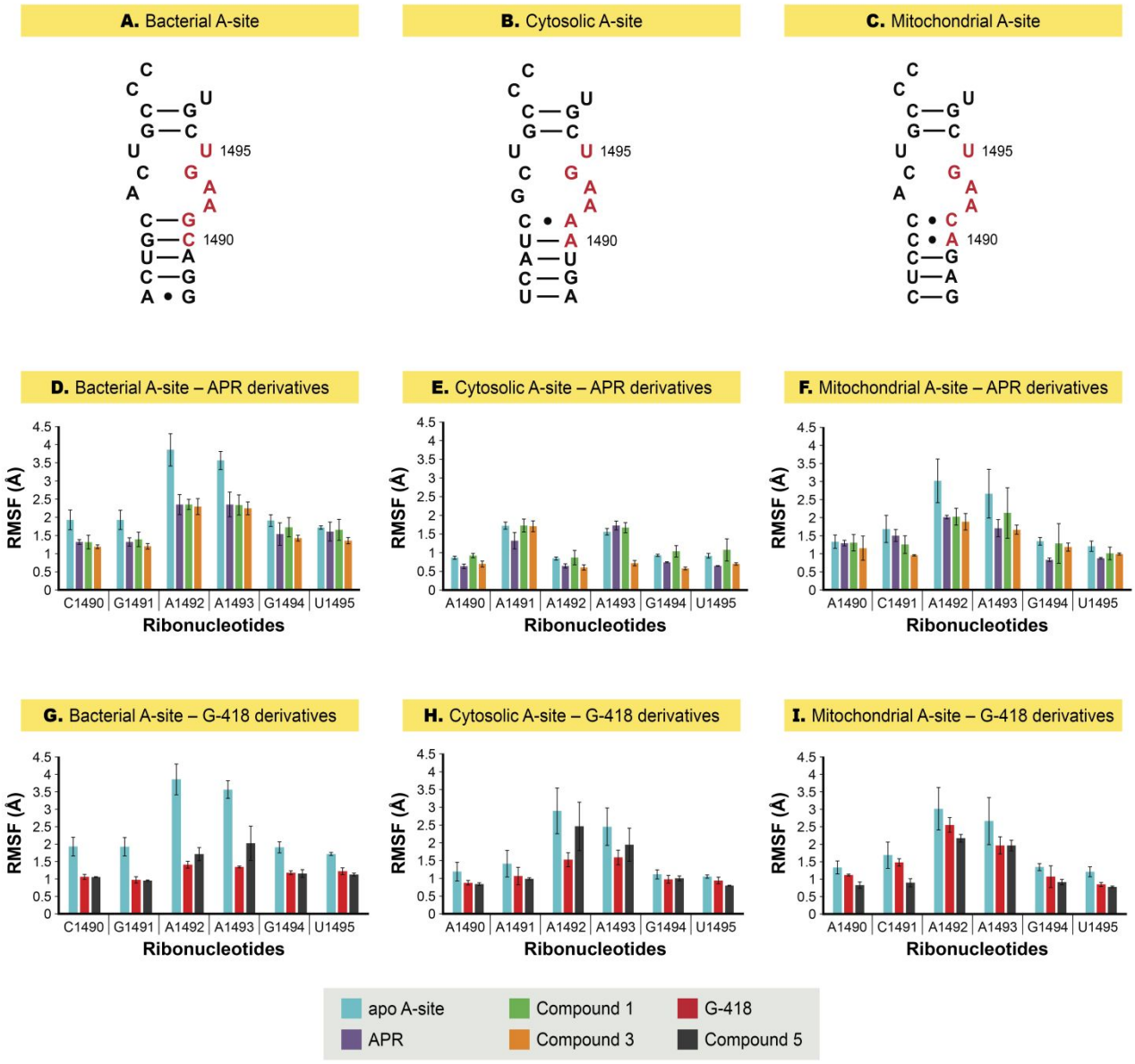

Figure 4. A-site secondary structures and the effects of APR, G-418, and compounds 1, 3, and 5 on the movement of ribonucleotides in the different A-sites. **(A-C)** Secondary structures of the **A**) bacterial, **B**) *H. sapiens* cytosolic, and **C**) *H. sapiens* mitochondrial A-sites. Ribonucleotides shown in red are those for which RMSF values were calculated. **(D-F)** RMSF values for apo A-sites and the A-sites in the presence of APR and its derivatives **1** and **3**. **(G-I)** RMSF values for apo A-sites and the A-sites in the presence of G-418 and its derivative **5**. RMSF values and standard deviations were determined from three independent simulations.

Compared to the previously reported structures, the best (lowest) docking scores were within 1.0 Å and 0.7 Å for APR (PDB code: 4AQY) and G-418 (PDB code: 1MWL), respectively, in the bacterial A-site and within 2.8 Å and 1.1 Å for APR (PDB code: 2G5K) and G-418 (PDB code: 4U4O), respectively, in the human cytosolic A-site (Section 4 in the Supporting Information). APR derivatives **1** and **3** and G-418 derivative **5** were then docked into the bacterial, cytosolic, and mitochondrial A-sites (Supporting Figure S2). Computations indicated that in the bacterial A-site, APR stabilized a conformation in which A1492 and A1493 are bulged out of the double helix; however, in the cytosolic A-site APR stabilized a conformation in which A1491 is bulged out, A1492 is intercalated into the helix, and A1493 is slightly bulged out. Complexes with the lowest docking scores were subjected to further analysis using MD simulations. For each complex, three 20-ns simulations were performed; 20 ns is the time required for the two adenines in the bacterial apo A-site to complete the movement between the bulged out and bulged in states.

Each replica was started from a different random seed. Simulations were probed every 10 ps to produce 2000 frames for each simulation. The stability of the resulting trajectories was tested based on the root mean square deviation (RMSD) values of the backbone atoms with respect to the equilibrated structures of the A-sites (Supporting Table S2).

To determine the degree of freedom of each ribonucleotide in each structure, the root mean square fluctuation (RMSF) values were calculated and compared to the corresponding values in the AG-bound A-sites (Figure 4D-I and Supporting Tables S3-S5). The lower the difference between the RMSF values of the ribonucleotides in the AG-bound sequence and the values of the corresponding ribonucleotides in the apo A-site, the lower the perturbation to the motion of the A-site. An optimal AG should significantly reduce the free movement of the ribonucleotides of the bacterial A-site but should not interfere with movement of the ribonucleotides in the eukaryotic A-sites.

The calculated RMSF values of A1492 and A1493 in the presence of APR and its derivatives **1** and **3** were similar in the bacterial A-site and lower than those in the bacterial apo A-site. These results suggest that the core APR scaffold confines the movement of A1492 and A1493 and that the added ribose moiety in **1** and **3** does not further reduce the movement of these two A-site ribonucleotides (Figure 4D).

MD simulations of the *H. sapiens* cytosolic A-site in the presence of APR and its derivatives **1** and **3** indicated that compared to the apo A-site, APR slightly reduced the freedom of movement of A1491 and A1492 yet, slightly increased the movement of A1493 (Figure 4E). Compared to the parent APR, compound **3**, the N-demethylated and ribosylated derivative, significantly constrained the movement of A1493. Of all AGs evaluated, ribosylated APR derivative **1** had the least effect on the movement of ribonucleotides of the eukaryotic cytosolic A-site.

In the mitochondrial A-site the RMSF values of A1492 were similar for APR and its derivatives **1** and **3** (Figure 4F). For A1493, the average RMSF value in the presence of **1** was higher than average RMSF values in the presence of APR and of **3**, suggesting that ribosylation of APR reduced the effect of this AG on the movement of the eukaryotic mitochondrial A-site ribonucleotides. Notably, the results of the MD simulations of APR and its derivative **1** in complex with the mitochondrial A-site sequence are consistent with the IC_{50} values of these AGs recently determined by analyses of bacterial ribosomes in which the native A-site was replaced by the 30-basepair sequence of the mitochondrial A-site; in this system, ribosylation of APR reduced its undesired inhibitory effect on cytosolic and mitochondrial A-sites.[62]

G-418 and its N-demethylated derivative **5** also significantly reduced the movement of adenines 1492 and 1493 of the bacterial A-site (Figure 4G). However, the RMSF values of **5** were slightly higher than those of G-418. By binding to the cytosolic A-site, G-418 reduced the movement of A1491, A1492, and A1493 with the largest difference in RMSF values calculated for the latter two adenines (Figure 4H). Notably, in agreement with the results of the cytosolic *in vitro* translation assay, the N-demethylated derivative **5** had a smaller effect on the freedom of movement of ribonucleotides in the cytosolic A-site than did G-418. The RMSF values for A1492, and A1493 when bound to **5** were higher than for G-418. Interestingly, G-418 and its N-demethylated derivative **5** had similar effects on the freedom of movement of A1493 in the mitochondrial A-site, but **5** reduced the freedom of movement of A1492 and of adjacent ribonucleotides C1491 and A1490 more than did the parent antibiotic G-418 (Figure 4I). The important role of the phylogenetic differences between ribonucleotides in proximity to A1492 and A1493, including ribonucleotide 1491, in facilitating the A-site selectivity of AGs has been established by Böttger and coworkers.[32,70]The different interactions of the AG with ribonucleotide 1491, which is a guanine in the bacterial A-site and a cytosine in the mitochondrial A-site (Figure 4A and 4C, respectively), appear to underlie the different effects of the AGs on the flexibility of the mitochondrial and bacterial A-sites.

In summary, MD simulations of the three A-sites indicated that the predominant effect of all AGs in this study was to reduce the freedom of movement of A1492 and A1493; however, some of the AGs also significantly affected the movement of ribonucleotides adjacent to these two adenines. Since there is higher sequence identity between the bacterial and human mitochondrial A-sites than between the bacterial and human cytosolic ones, we had expected that the AGs would have similar effects on the ribonucleotides of the bacterial and mitochondrial A-sites: The MD simulations suggested otherwise.

Ribosylation and N-demethylation reduce the effect of AGs on the viability of HEI-OC1 cells. We next evaluated the viability of immortalized cells derived from inner ear auditory cells in the presence of the most potent antibacterials of the AGs studied here; the results are summarized in Figure 5. As an inner ear cell model, we used HEI-OC1 cells, which are immortalized mouse inner ear cells that express markers of auditory sensory cells. This cell line, developed by Kalinec and co-workers, has become a widely used model for the study of the damage induced by AGs on auditory sensory cells.[71,72]

At the highest concentration tested, APR and its two derivatives **1** and **3** did not measurably decrease the viability of HEI-OC1 cells (IC50 >> 5 mg/mL, Figure 5). G-418 was highly toxic to the cells with an IC50 value of about 0.2 mg/mL. Notably, the IC50 value of **5** was about 1.8 mg/mL, which is higher by close to an order of magnitude compared to that of the parent antibiotic. The effects of the tested AGs on the viability of HEI-OC1 cells correlated well with their effects on cytosolic translation *in vitro*. Less potent inhibitors of translation in the rabbit reticulocyte lysates were also the less toxic to HEI-OC1 cells. For example, G-418 inhibited eukaryotic cytosolic translation over an order of magnitude more potently than its demethylated derivative **5** (Figure 3); the parent AG was

about an order of magnitude (~9-fold) more toxic to HEI-OC1 cells than **5** (Figure 5).

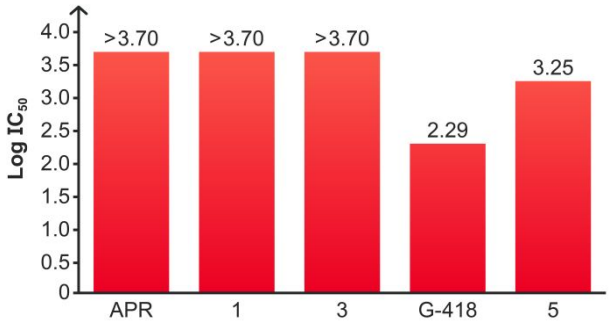

Figure 5. Effects of APR, G-418, and compounds 1, 3, and 5 on the viability of HEI-OC1 cells. Cells were treated with different concentrations of each compound for 72 h, and viability was determined using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay; $\log_{10}$ scale IC_{50} values plotted are relative to untreated control cells. Each concentration was tested in duplicate, and the results are expressed as $\log_{10}$ of the IC_{50} values from two independent experiments.

The maximal concentration of AGs in human plasma (C_{max}) depends on numerous parameters such as the AG's chemical structure and overall positive charge, the administration regime, the dose, and the patient's physiology including weight and kidney function. Based on several clinical reports, the C_{max} values of clinically used AGs in human plasma are in the range of 10 to 100 μg/mL.[47–50] Interestingly, this suggests that, with an IC_{50} value of about 200 μg/mL, G-418 is the only AG in this study that would reach the cochlea at a concentration sufficient to induce significant toxicity to the auditory hair cells.

Chemical modifications affect the abilities of the AGs to permeabilize HEI-OC1 cells. We next asked if the AGs APR and G-418 induce rapid permeabilization of HEI-OC1 cells and if chemical modifications affect this phenomenon. To evaluate the permeability of HEI-OC1 cells, we measured the uptake of 4′,6-diamidino-2-phenylindole (DAPI), which is excluded from healthy cells due to the integrity of the plasma membrane. Uptake of DAPI was measured after a 3-hour incubation with the tested AGs using flow cytometry. As a positive control we chose a membrane-disrupting cationic amphiphile derived from the AG tobramycin (S-14) previously developed by our group; S-14 induces rapid and extensive plasma membrane damage.[73] As a negative control, cells were exposed to the translation-inhibiting antibiotic chloramphenicol (CAM).[74] We quantified DAPI-free cells, cells stained with DAPI, and dead cells. Results are summarized in Figure 6 and in Figure S3.

Compared to untreated cells, a dose-dependent increase in the percentage of permeabilized cells was observed in samples treated with APR. This increase was moderate compared to that induced by G-418. Interestingly, compared to G-418-treated cells, a substantially lower percentage of cells were DAPI stained in samples treated with N-demethylated derivative **5**. Following a 3-hour exposure to 300 μg/mL of **5**, the percentage of DAPI-stained cells was about an order of magnitude lower than that of cells treated with 300 μg/mL of G-418 and slightly lower than that of cells treated with 300 μg/mL of APR (Figure 6).

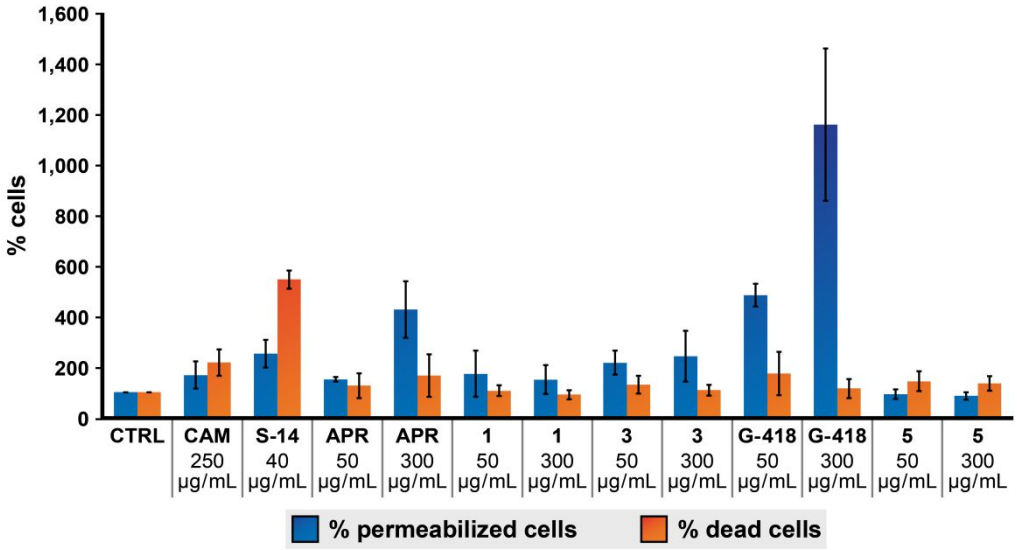

Figure 6. Effects of APR, G-418 and compounds 1, 3 and 5 on the permeabilization of HEI-OC1 cells. Cells were treated with different concentrations of each compound for 3 h, and permeability was evaluated by flow cytometry analysis of DAPI staining. The percentages of permeabilized and dead cells were determined relative to untreated cells. CAM was used as negative control. Dead cell population was determined using the parameters measured for cells treated with cationic amphiphile S-14 that causes rapid and extensive loss of membrane integrity. Results are expressed as means ± SD from two independent experiments.

The results of the cell permeabilization experiments indicate that, depending on the structure, AGs can induce rapid plasma membrane damage. Importantly, AGs enhanced DAPI permeability after a relatively short incubation of 3 hours, suggesting that the enhanced permeability is unlikely to result only from the accumulation of dysfunctional proteins. Rather, we speculate that permeabilization mainly results from direct plasma membrane disruption by these highly positively charged molecules. Thus, the results of the cell permeabilization assay suggest that, depending on the AG, the mechanism leading to auditory cells damage may vary and that even minor chemical modifications affect the mode of action leading to cell damage.

AG structure influences the levels of mitochondrial proteins translated by cytosolic and mitochondrial ribosomes in HEI-OC1 cells. Current techniques for analysis of *in vitro* translation are based on cell-free assays, and it is technically difficult to generate extracts of mitochondrial ribosomes devoid of contaminating cytosolic ribosomes. We therefore developed an experiment to study the effects of AGs on the levels of mitochondrial proteins translated by mitochondrial and cytosolic ribosomes in intact cells. This was accomplished by repurposing a flow cytometry assay that was originally designed to evaluate drug-induced effects on mitochondrial biogenesis. This assay relies on the statistical power of flow cytometry to evaluate the ratio between two mitochondrial proteins, one encoded by mitochondrial DNA and translated by mitochondrial ribosomes (COX-1) and the second encoded by nuclear DNA and translated by cytoplasmic ribosomes (SDHA). As a positive control for the assay we used CAM, which inhibits mitochondrial translation.[75]

Briefly, HEI-OC1 cells were treated with AGs for 6 days at concentrations around the reported plasma C_{max} values of AGs (10, 50, 100, and 300 µg/mL). Cells were then harvested, fixed, and permeabilized in suspension. COX-1 and SDHA were detected by flow cytometry with highly specific monoclonal antibodies labeled with two different fluorophores (Figure 7 and Figure S4). This protocol minimizes sample preparation and handling to enable evaluation of the effects of the tested AG on the mitochondrial and cytosolic protein levels.

In samples treated with 25 µg/mL of the control antibiotic CAM, there was a reduction in the level of COX-1, which is translated in mitochondria, as compared to untreated control samples (Figure 7). This is in agreement with the previous observation made by Kang et al. that COX-1 levels are reduced in CAM-treated cells.[76] In contrast to the effect on COX-1, treatment with CAM led to a dose-dependent increase in the level of SDHA, a mitochondrial protein translated by cytosolic ribosomes (Figure 7). This unexpected effect may be the result of activation of a stress-response mechanism; it was previously shown that shear stress in mammalian cells leads to elevated levels of SDHA.[77]

Paradoxically, the AGs that effectively inhibited both bacterial and mammalian translation *in vitro* increased levels of both SDHA and COX-1 in HEI-OC1 cells; the extent of elevation depended on the concentration and on the AG. Only a modest dose-dependent increase in the levels of COX-1 and of SDHA was measured in cells treated APR or with its derivatives **1** or **3** (Figure 7). In cells treated with G-418, the dose-dependent increase in COX-1 and SDHA levels was higher compared to that measured for APR and its derivatives. Compared to G-418, 100 µg/mL of **5**, the N-demethylated derivative of G-418, had much less effect.

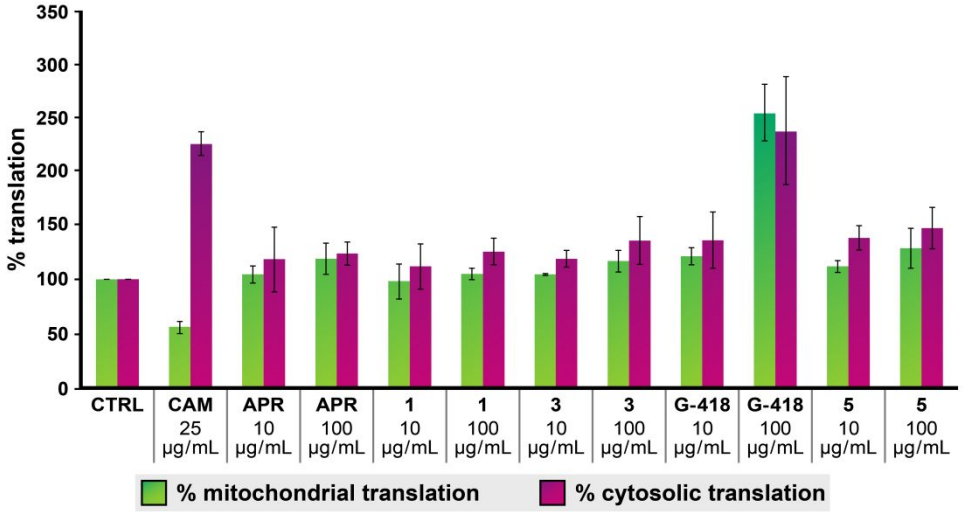

Figure 7. Effects of APR, G-418, and compounds 1, 3, and 5 on levels of mitochondrial proteins translated in the cytoplasm and the mitochondria of HEI-OC1 cells. Cells were treated with different concentrations of each compound for 6 days, and levels of COX-1 (translated in the mitochondria, in green) and SDHA (translated in the cytoplasm, in magenta) were determined by flow cytometry. The percentages of protein levels relative to untreated cells are plotted. CAM was used as a positive control for inhibition of mitochondrial translation. The results are expressed as means ± SD from two independent experiments.

Interestingly, the elevation of COX-1 and SDHA levels induced by the different AGs correlated with their effects on the viability of HEI-OC1 cells. For example, of the AGs tested in this study, G-418 was the most toxic to HEI-OC1 cells (IC50 about 200 μg/mL, Figure 5), and it also induced the largest increase in COX-1 and SDHA levels (Figure 7). N-demethylated G-418 derivative **5** was about 9-fold less toxic to HEI-OC1 cells than the parent AG, and the increases it induced in levels of the proteins translated in the mitochondria and the cytosol were considerably lower than that of the parent AG. The results of these assays counterintuitively suggest that, when introduced at clinically relevant concentrations, AGs may enhance rather than reduce the levels of certain proteins in mammalian cells. This is likely the result of a stress response and does not contradict the well-established paradigm that AGs perturb with both bacterial and eukaryotic translation processes.

CONCLUSIONS

Here we investigated how known, as well as novel chemical modifications of APR, considered among the least toxic of the AGs, and G-418, considered the most toxic, influenced different aspects of their biological activity with a focus on their effects on auditory cells. Interestingly, for APR and G-418, N-demethylation and ribosylation resulted in opposite effects on the antibacterial activity. Conversion of the single secondary amine of APR to a primary amine through N-demethylation reduced its antibacterial activity; the effect of this modification on the antibacterial activity of G-418 was significantly less pronounced. Ribosylation, in contrast, did not considerably alter the antibacterial activity of APR yet completely abrogated that of G-418. Comparison of the effects of these AGs on inhibition of bacterial versus eukaryotic cytosolic translation revealed that N-demethylation increased the selectivity of G-418 by more than an order of magnitude.

MD simulations of complexes between the AGs and rRNA fragments corresponding to bacterial, eukaryotic cytosolic, and eukaryotic mitochondrial A-sites showed that binding to AGs rigidified the A-site structures reducing the freedom of movement necessary for accurate translation. A correlation was found between the extent of restriction of movement and the inhibition of cell-free translation. AGs that effectively inhibited eukaryotic and/or bacterial translation processes also effectively reduced the movement of the corresponding A-sites. In the A-sites, the positions of the adenines that define the on and off states and those nucleotides in close proximity to these adenines were affected more than other nucleotides in the A-sites by AG binding. MD simulations indicated that even minor modification to the AG scaffold, such as N-demethylation, significantly altered the effect of the AG on the flexibility of the A-site. Further, differences in A-site sequences, such as those that differentiate between the mitochondrial and bacterial A-sites, resulted in differences in interactions with AGs. The *in silico* analyses proved a powerful tool for prediction of the effects of AGs on bacterial and mammalian cell translation processes, thereby paving the way for rational design of additional AG derivatives.

Even at concentrations one to two orders of magnitude higher than the reported maximal plasma concentrations range of AGs in various mammals, including humans, APR and its ribosylated derivatives **1** and **3** did not affect the viability of HEI-OC1 cells. These immortalized mouse inner ear cells express markers of auditory sensory cells. Notably, although it is a relatively minor chemical modification, N-demethylation of the highly toxic G-418 markedly reduced its toxicity to HEI-OC1 cells; the N-demethylated derivative **5** inhibited viability of HEI-OC1 cells by 50% at a concentration close to an order of magnitude higher than the IC50 of the parent AG G-418.

All AGs in this study inhibited both the bacterial and eukaryotic cytosolic ribosomes in cell-free experiments; however, a different picture appeared when additional effects of these antibiotics on HEI-OC1 cells were studied. A dose-dependent enhancement in uptake of DAPI by HEI-OC1 cells after only 3 hours of incubation with the AGs was indicative of a rapid plasma membrane permeabilization effect. The results of the DAPI uptake assay indicated that this effect could be significantly reduced through chemical modifications. For example, a considerably lower percentage of cells were permeable to DAPI upon treatment with **5**, the N-demethylated derivative of G-418, than upon treatment with the parent antibiotic.

By concomitant flow cytometry analyses of the levels of COX-1, a protein translated by the mitochondrial ribosome, and SDHA, which is produced in the cytoplasm, we observed that, at clinically relevant concentrations, the AGs tested enhanced, rather than reduced, the levels of these mitochondrial proteins in HEI-OC1 cells with the most significant dose-dependent enhancement measured in cells treated with G-418. This seemingly paradoxical effect may be due to a stress response. Importantly, these results emphasize the need for further investigation of the role of the stress response in AG-induced auditory cell damage.

To summarize, this study demonstrated that despite common chemical features, the vast structural diversity among different members of the AG class of antibiotics results in major differences in desirable and undesirable biological activities. All AGs tested inhibited the activities of mammalian and bacterial ribosomes when tested in cell-free assays, albeit with different efficacies; however, our data suggest that, depending on the AG, toxicity to auditory cells may stem from rapid plasma membrane permeabilization. Moreover, although AGs are potent inhibitors of translation in cell-free extracts, in intact cells these drugs can cause an elevation in the levels of certain proteins, presumably, due to stress responses to these antibiotics. Importantly, this study demonstrated that chemical modifications can improve selectivity of AGs as well as reduce plasma membrane permeabilization of auditory cells. The synthetic strategies and approaches for biological and *in silico* evaluation described should lead to

breakthroughs in development of AGs with reduced toxicity to auditory cells.

ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures; MIC assay protocol and MIC values (Table S1); in vitro translation protocols; docking and MD calculation protocols; RMSD values (Table S2); RMSF values(Tables S3-S5); cytosolic A-site sequence used for MD simulations (Figure S1); docked structures of the AGs in the different A-sites (Figure S2); HEI-OC1 cell viability assay protocol; cell permeability assay protocol (Figure S3); protocol for in-cell mitochondrial and cytosolic protein translation (Figure S4); ^{1}H and ^{13}C NMR assignments for intermediate compounds and final compounds **1-9**; MS data; ^{1}H and ^{13}C NMR spectra of compounds **1-9** (Figures S5-S22).

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ACKNOWLEDGMENT

We thank Dr. Federico Kalinec (House Ear Institute, Los Angeles, California) for his generous gift of HEI-OC1 cells and for his advice. We thank Dr. Reuven Stein (Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel) for his guidance and access to the mammalian cell culture facility. This work was supported by Israel Science Foundation Grant 6/14.

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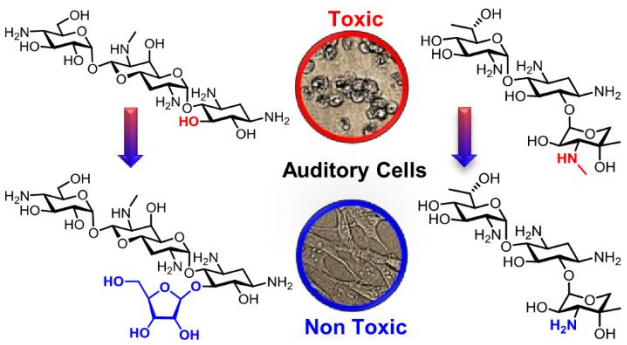
TABLE OF CONTENT ARTWORK



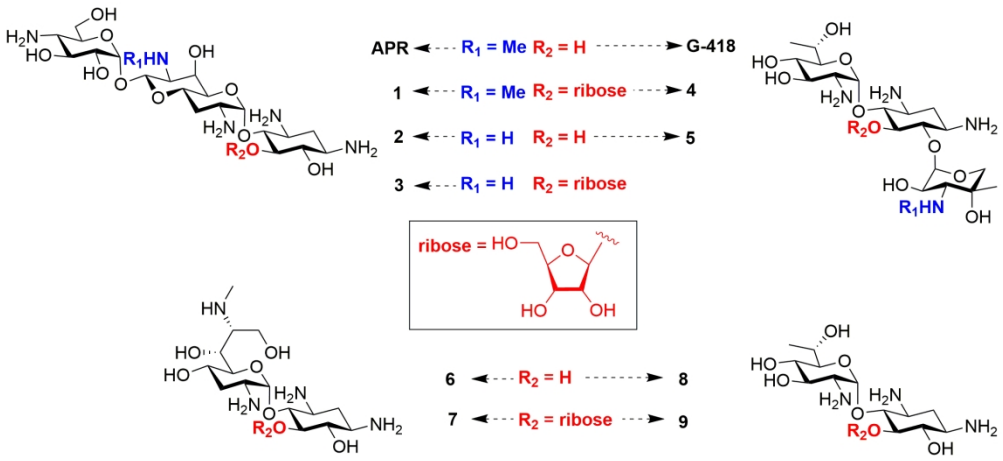


Figure 1: Structures of the AGs APR and G-418 and their semisynthetic derivatives.

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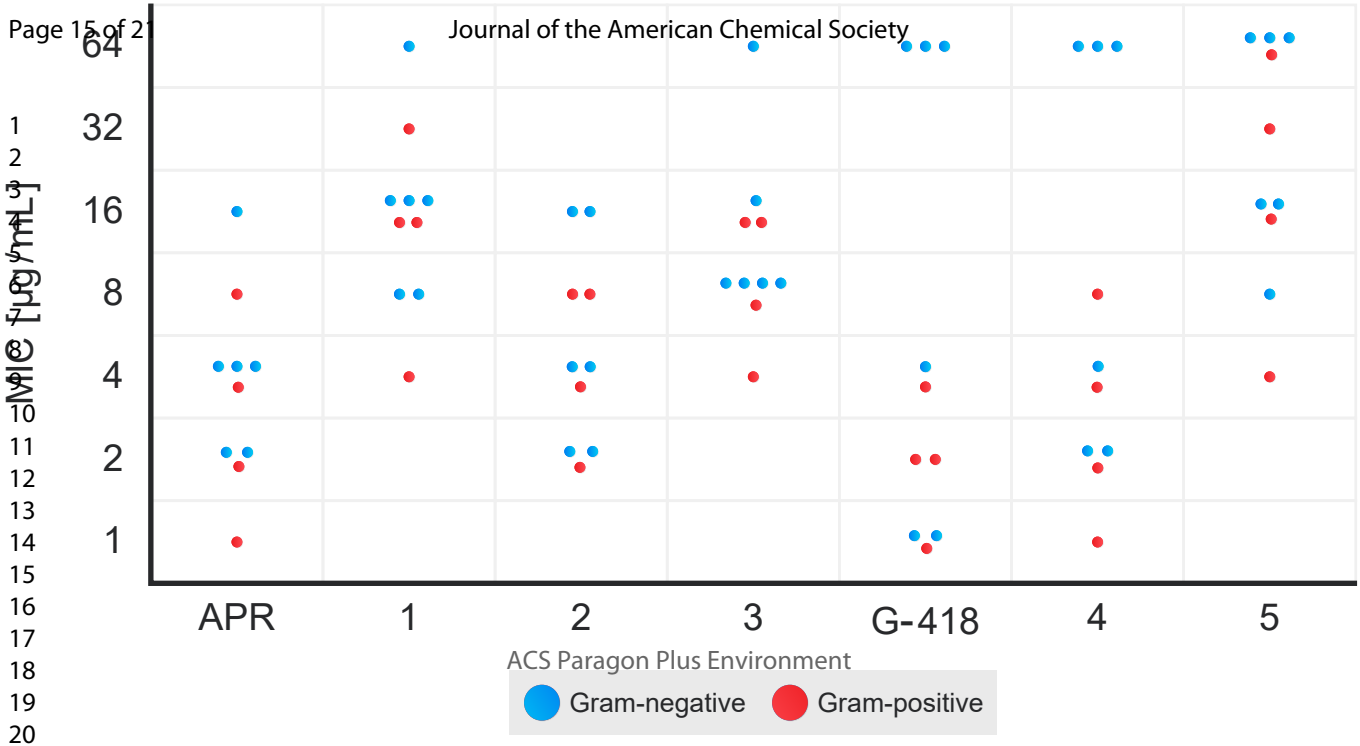
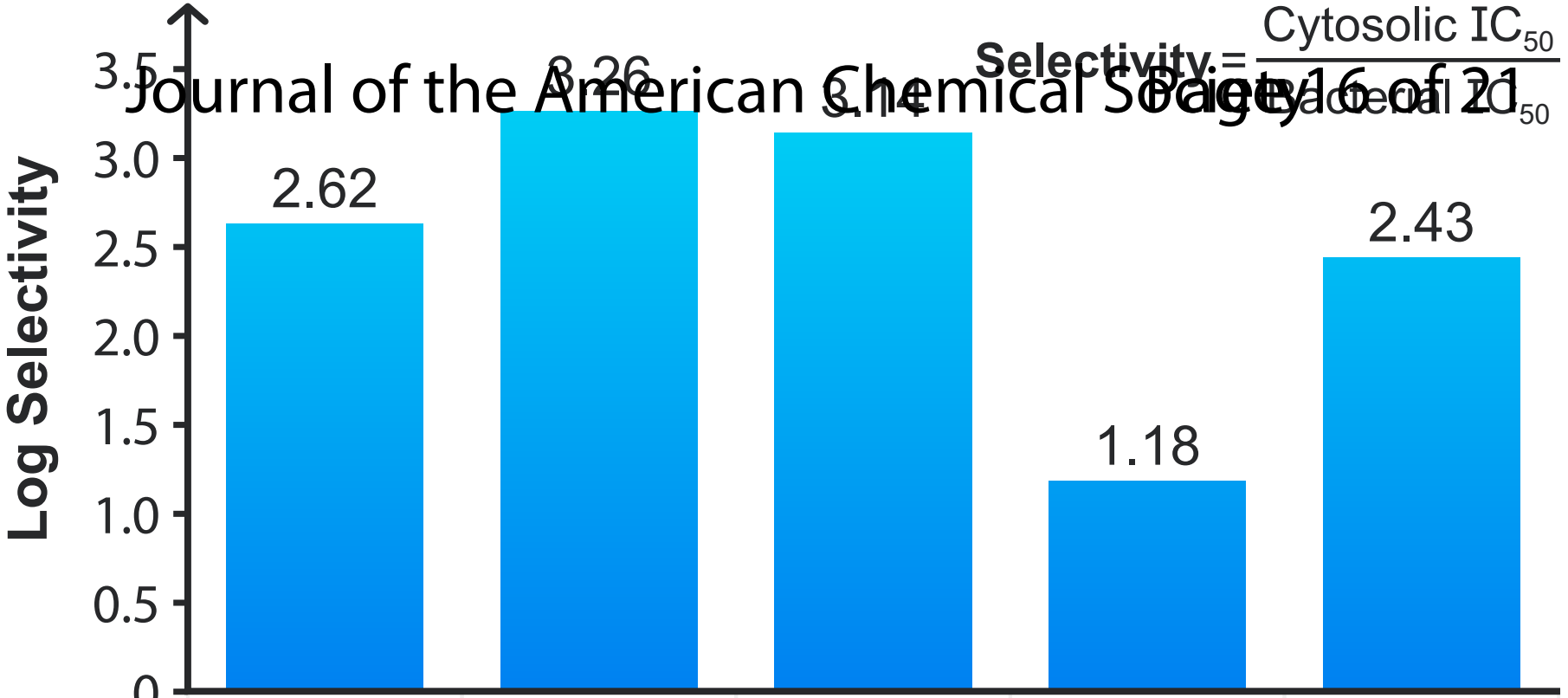
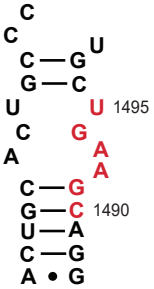

MIC [μg/mL]
64
32
16
8
4
2
1
APR
1
2
3
G-418
4
5
ACS Paragon Plus Environment
Gram-negative
Gram-positive



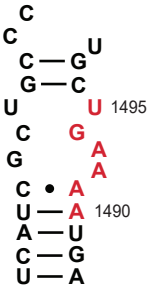
	APR	1	3	G-418	5
Cytosolic IC_{50} [a]	42.0±3.0	72.3±9.2	109.3±17.8	0.3±0.05	5.4±0.2
Bacterial IC_{50} [a]	0.10±0.02	0.04±0.0	0.08±0.0	0.02±0.0	0.02±0.0

[a] IC_{50} units are in [μM]

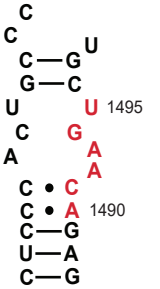
A. Bacterial A-site



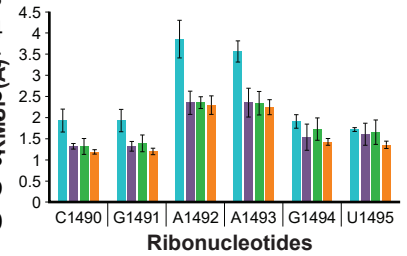
B. Cytosolic A-site



C. Mitochondrial A-site



D. Bacterial A-site – APR derivatives



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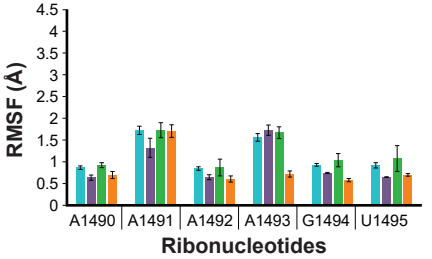
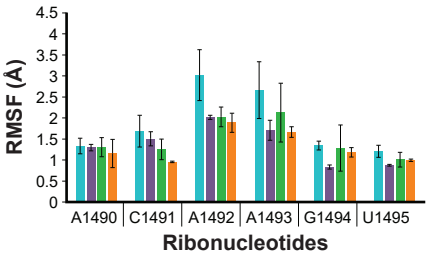
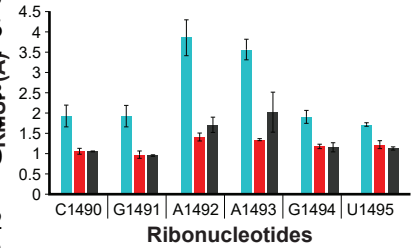


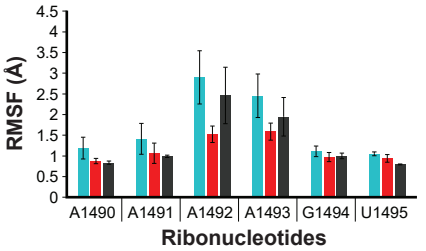
F. Mitochondrial A-site – APR derivatives



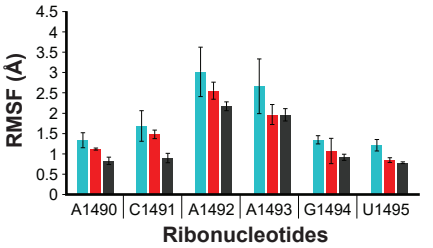
G. Bacterial A-site – G-418 derivatives

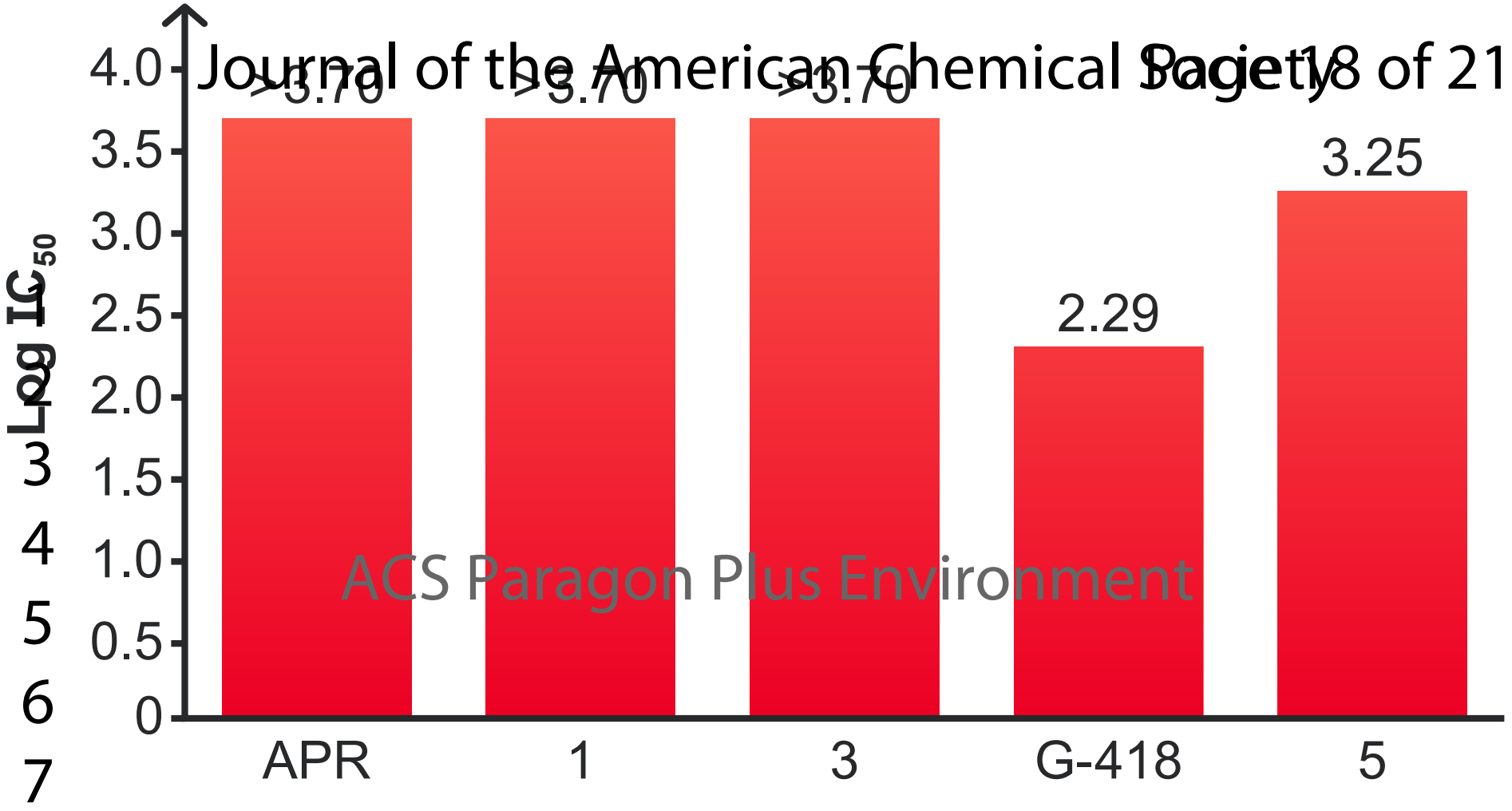


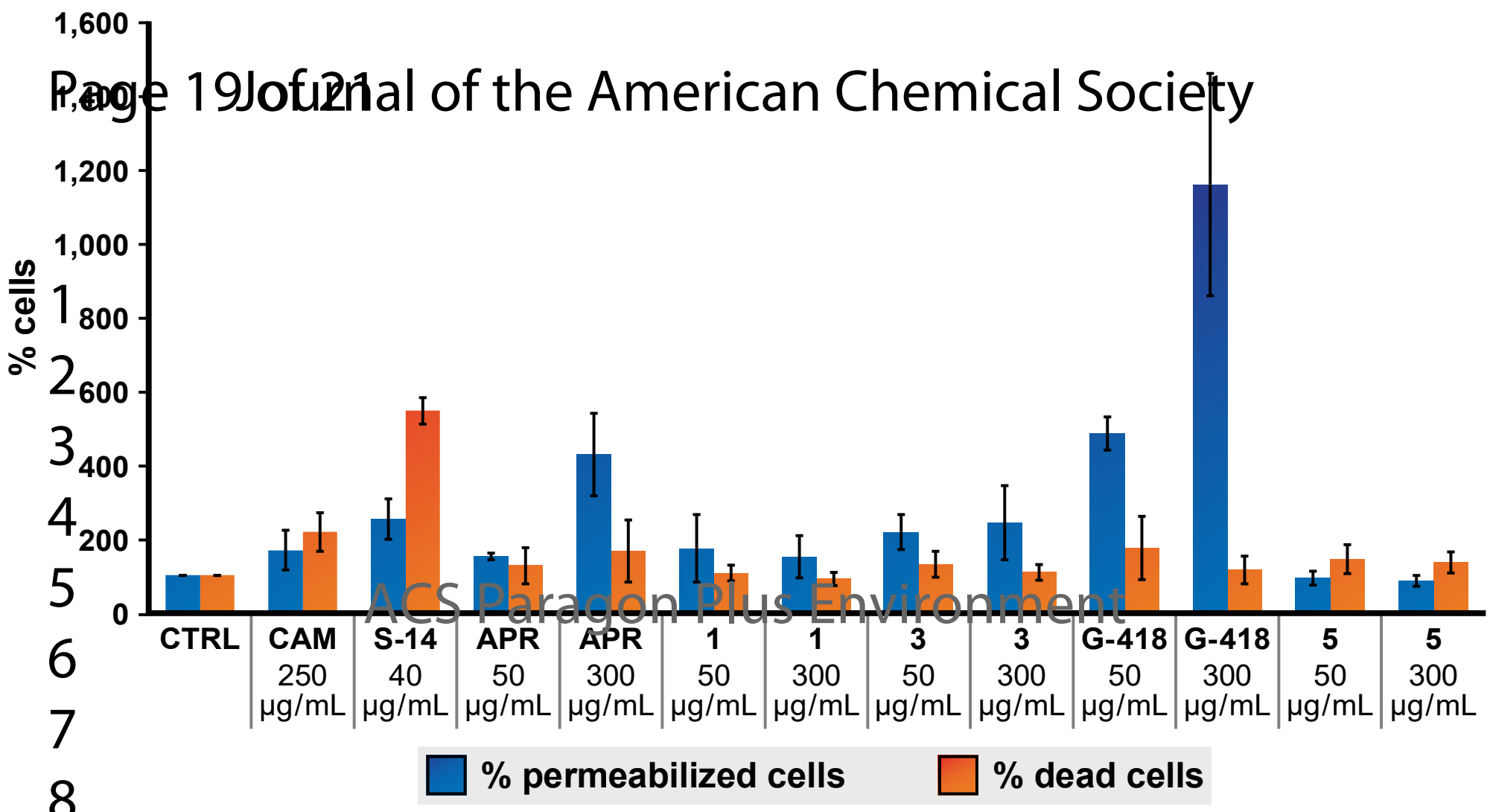
H. Cytosolic A-site – G-418 derivatives

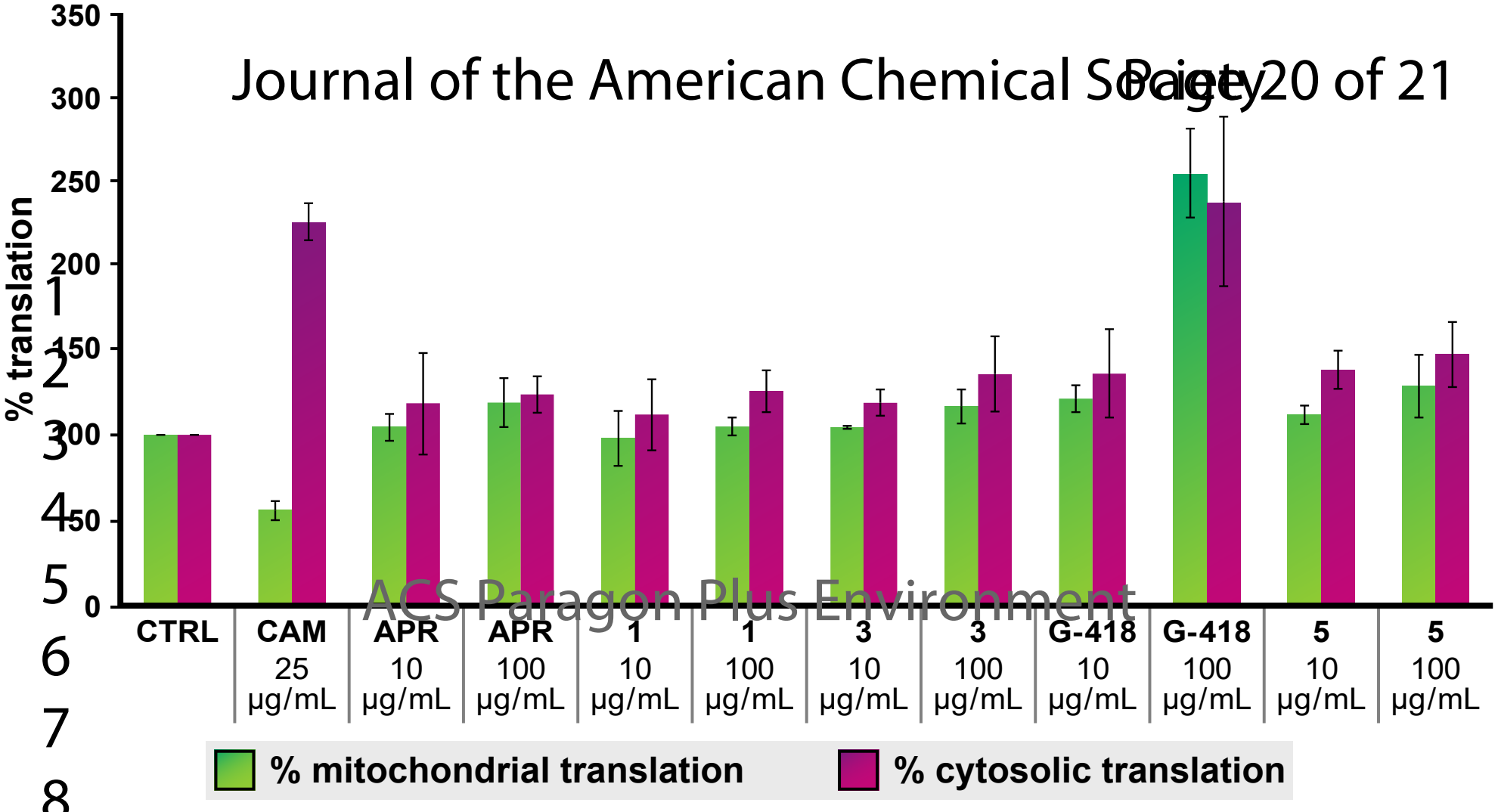


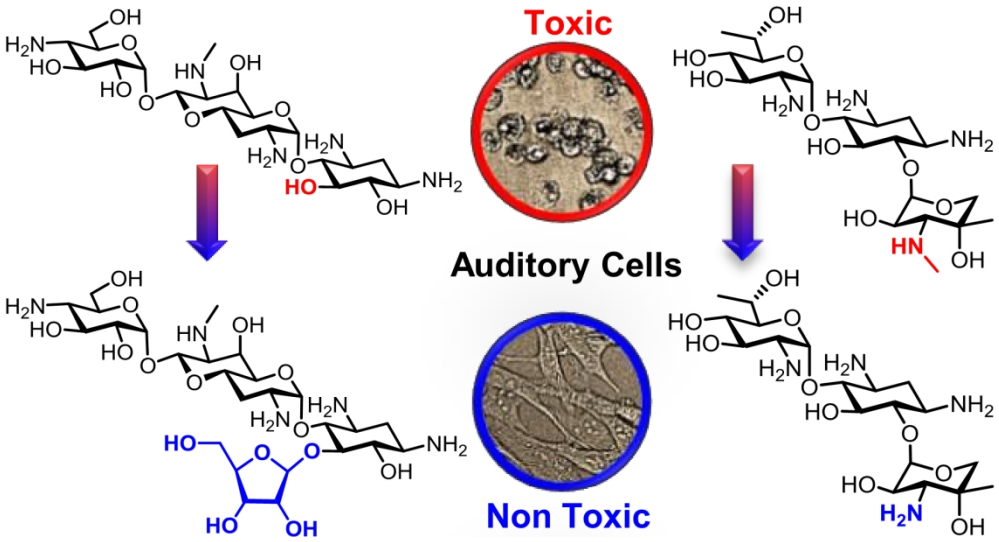
I. Mitochondrial A-site – G-418 derivatives











Art-work for Table of Content

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