Bioorganic & Medicinal Chemistry 18 (2010) 3735–3746

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Repairing faulty genes by aminoglycosides: Development of new derivatives of geneticin (G418) with enhanced suppression of diseases-causing nonsense mutations

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ARTICLE INFO

Article history:
Received 8 February 2010
Accepted 25 March 2010
Available online 27 March 2010

Dedicated to 'Tetrahedron Young Investigator 2010: Peter Seeberger'

Keywords:
Aminoglycosides
Genetic diseases
Nonsense mutations
Translational therapy
Cystic fibrosis

ABSTRACT

New pseudo-di- and pseudo-trisaccharide derivatives of the aminoglycoside drug G418 were designed, synthesized and their ability to readthrough nonsense mutations was examined in both in vitro and ex vivo systems, along with the toxicity tests. Two novel lead structures, NB74 and NB84, exhibiting significantly reduced cell toxicity and superior readthrough efficiency than those of gentamicin, were discovered. The superiority of new leads was demonstrated in six different nonsense DNA-constructs underling the genetic diseases cystic fibrosis, Duchenne muscular dystrophy, Usher syndrome and Hurler syndrome.

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1. Introduction

The treatment of genetic disorders is one of the biggest challenges lying ahead of modern medicine.[1–3] There are more than 1800 inherited human diseases caused by nonsense mutations, that is, alterations in the genetic code that prematurely stop the translation process and lead to the production of truncated non-functional proteins. These false stop codons are also known as premature termination codons (PTCs) and, depending on the disorder, account for 5–70% of cases of genetic diseases, including cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), Usher syndrome (USH), Hurler syndrome (HS) and numerous types of cancer. For many of those diseases there is presently no effective treatment and only treatment widely used is symptomatic. While major advancements have been made in gene therapy, it is still far from achieving clinical success. However, other potential methods for treating single gene related diseases have also emerged during last decades. One such approach is the suppression of pathogenic nonsense mutations through inducing translational readthrough of the in-frame PTCs.[2,3] Aminoglycoside antibiotics were the first drugs that gave promising results in this respect: numerous in vitro and in vivo experiments including clinical trials have demonstrated the ability of certain types of aminoglycosides to induce mammalian ribosome to readthrough disease-causing PTC mutations and partially restore full-length functional proteins.[1,3]

The fact that aminoglycosides (namely paromomycin and G418, Fig. 1) could suppress PTC mutations in cultured mammalian cells was first demonstrated by Burke and Mogg in 1985, who also pointed out the therapeutic potential of these drugs in treatment of genetic disorders.[4] The first genetic disease examined was CF that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein; nonsense mutations found in the *CFTR* gene could be suppressed by aminoglycosides G418 and gentamicin (Fig. 1), as measured by the appearance of full-length, functional CFTR protein in bronchial epithelial cell lines.[5,6] Further experiments with gentamicin, both in mouse models of CF[7] and DMD[8] and in clinical trials with patients of these two diseases[9,10] carrying nonsense mutations in *CFTR* and *Dystrophin* genes, respectively, evidently demonstrated partial production of missing proteins. Other genetic disorders for which the therapeutic potential of aminoglycosides was tested in in vitro systems, cultured cell lines, or animal models including cancer,[11] Rett syndrome,[12] Hurler syndrome,[13] nephrogenic diabetes insipidus,[14] nephropathic cystinosis,[15] retinitis pigmentosa,[16] ataxia-telangiectasia[17] and more.

It should be noted that aminoglycoside-induced production of full-size protein in the presence of a PTC mutation, even if only in low amount, may be functionally significant. This is especially

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doi:10.1016/j.bmc.2010.03.060

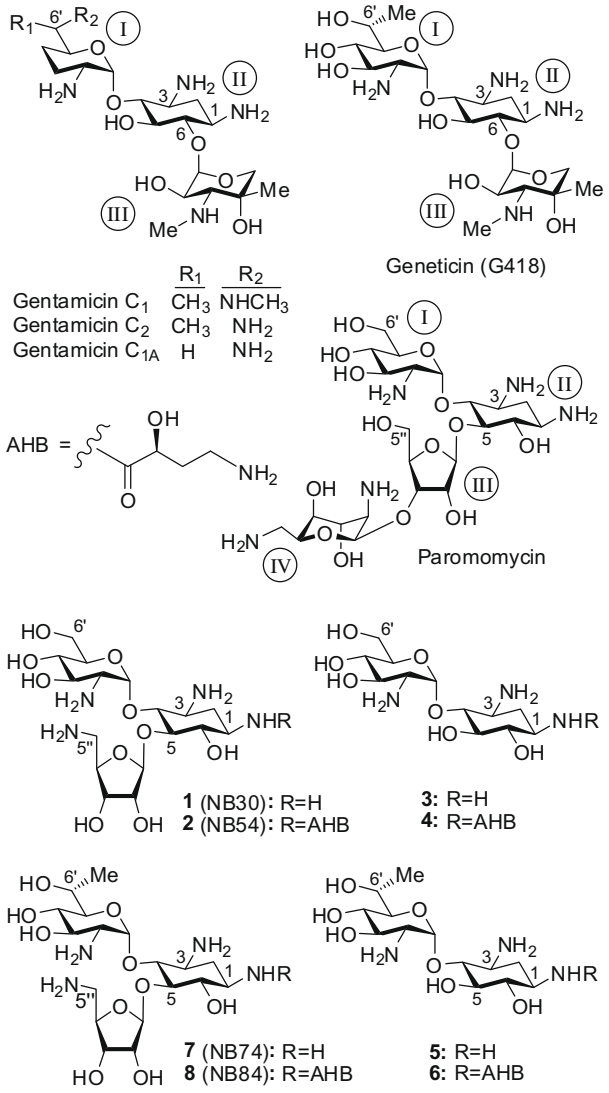


Figure 1. Chemical structures of a series of natural and synthetic 2-deoxystreptamine-derived (ring II) aminoglycosides, including compounds **1–8** that were investigated in this study.

relevant for recessive disorders, where protein expression is essentially absent, like CF, DMD, HS, ataxia-telangiectasia and others, and even 1% of normal protein function may restore a near-normal or clinically less severe phenotype.[3,9,18] Consequently, the greatest promise of aminoglycosides as potential pharmacogenetic agents is primarily for the treatment of recessive disorders caused by nonsense mutations. Recent studies have indicated that aminoglycosides induced readthrough may be a promising therapy in autosomal-dominant disorders as well.[19]

Despite this great excitement and promise that continues over the last two decades, severe side-effects of aminoglycosides including high toxicity to mammals along with the reduced readthrough efficiency at subtoxic doses, have limited their clinical benefit for suppression therapy.[20] In addition, to date, the clinical application of aminoglycosides is limited to their use as antibiotics, and almost no efforts have been made to optimize their activity as stop codon readthrough inducers. Furthermore, while the recent X-ray crystal structures of the bacterial ribosome in complex with aminoglycosides shed light on the mechanism of aminoglycosides action as antibiotics,[21,22] no crystal structure of human ribosome is presently available and the mechanism of aminoglycoside-induced readthrough is largely obscure.[23,24] Clearly, the challenges in this emerging field should be at deciphering the intimate structural and functional determinants of aminoglycosides as readthrough and toxicity inducers, in order to develop new structures with improved suppression activity and reduced toxicity.

Towards these ends, we have recently reported on the development of compound **1** (also named NB30, Fig. 1)[25] and compound **2** (also named NB54, Fig.1)[26] as novel pseudo-trisaccharide derivatives of the clinical aminoglycoside paromomycin. Whereas the first-generation lead, compound **1**, exhibited significantly reduced cytotoxicity in comparison to gentamicin and paromomycin,[27] and promoted dose-dependent suppression of nonsense mutations of the *PCDH15* gene, one of the underlying causes of type 1 Usher syndrome (USH1),[27] its suppression potency was significantly lower relative to that of gentamicin and paromomycin. In attempts to further improve the suppression efficiency and reduce the toxicity of **1**, we have developed the compound **2** as a second-generation lead structure.[26] Compound **2** exhibited significantly reduced cell, cochlear and acute toxicities, and has substantially higher stop-codon readthrough potency in both in vitro and ex vivo studies, than those of gentamicin and paromomycin.[26] Continuing systematic fine-tuning of the developed leads, in the present work we report on the design, synthesis and comparative evaluation of the third-generation lead structures **7** and **8** (also named NB74 and NB84, respectively, Fig. 1). They differ from the previous leads (**1** and **2**) by the presence of the (*R*)-6′-methyl group on the glucosamine ring (ring I) and show significantly reduced cell toxicity while in parallel exhibit substantially higher stop-codon readthrough potency in both in vitro and ex vivo studies, than those of gentamicin. The observed comparative data also indicate that the introduction of (*R*)-6′-methyl group either on compound **1** (to give compound **7**) and on compound **2** (to give compound **8**) greatly enhances the stop-codon readthrough potency of the resulted structure (compounds **7** and **8**, respectively), while its effect on cell toxicity of the resulted compounds in comparison to those of the parent structures was negligible.

2. Results

2.1. Design hypotheses and synthesis

To further improve both the premature stop codon suppression activity and toxicity profiles of **1** and **2**, we sought to explore the effect of selective installation of the (*R*)-6′-methyl group on these structures to yield compounds **7** and **8**, respectively, because of the following reasons. First, although to date there are not enough structural and mechanistic data available to answer the question why some aminoglycosides induce termination suppression in mammalian systems while others do not, from the existing data it is generally accepted that aminoglycosides with a C6′ hydroxyl group on ring I (such as paromomycin and G418, Fig. 1) are more effective than those with the amine functionally at the same position.[28–30] Second, among all aminoglycosides tested to date for readthrough activity,[31,32] G418 is the only one that has (*R*)-6′-methyl group on its glucosamine moiety (ring I) with the secondary alcohol at 6′ position. Furthermore, G418 is also the strongest readthrough inducer among all aminoglycosides tested to date[5,6,31] and no detailed SAR study on its derivatives has yet been performed. Third, despite its potent readthrough activity, the use of G418 as a therapeutic agent is not possible because it is lethal even at very low concentrations.[33] However, it is not known what structural element(s) of G418 contribute to its elevated readthrough activity and what to its high cytotoxicity. Separation of such elements, if possible, should be of high importance for the development of new derivatives with potent suppression activity and

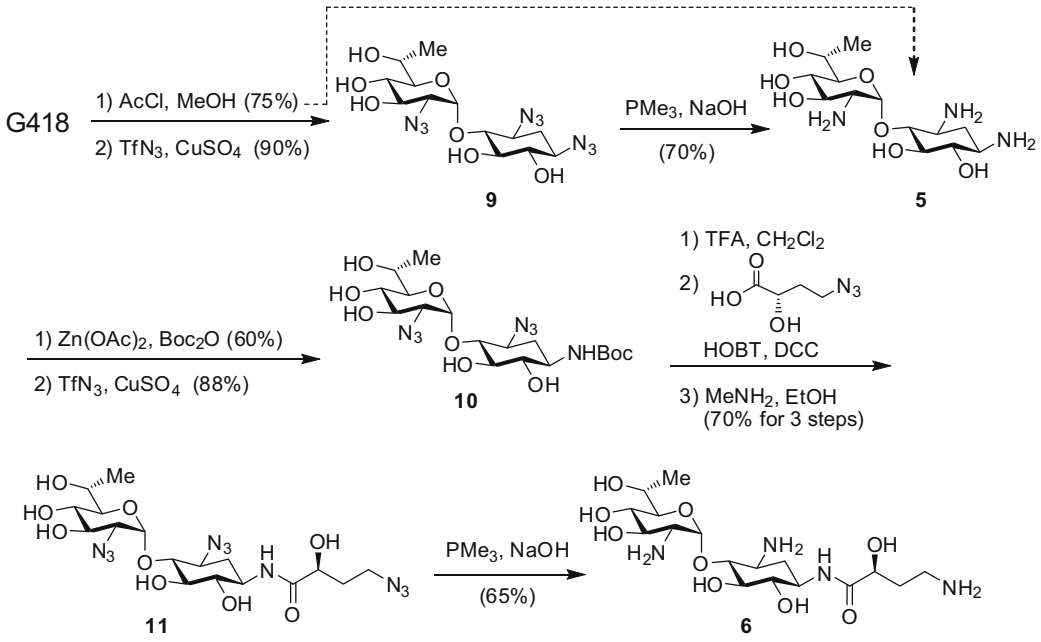
reduced toxicity. For example, very recent study on gentamicin demonstrated that the inversion of an absolute configuration at a single carbon atom, from (*S*)-6′-gentamicin C_2 to (*R*)-6′-gentamicin C_2, significantly reduces cell toxicity and apparent nephrotoxicity of the (*R*)-diastereomer in comparison to that of (*S*)-diastereomer, as determined in cell culture and animal studies, while the bactericidal efficacy is not affected.[34] Based on these accumulative data, we anticipated that the exploration of the impact of (*R*)-6′-methyl group could lead to new derivatives of aminoglycosides with improved readthrough activity and probably with reduced toxicity.

Initially, the concept was tested on pseudo-disaccharides; we synthesized compounds **3–6** (Fig. 1) and tested their comparative in vitro readthrough efficiency. Compounds **3** and **4** were synthesized as previously reported by us.[25,26] As a starting material for the synthesis of **5** and **6** we used G418 (Scheme 1). Treatment of G418 with anhydrous HCl (AcCl in MeOH) gave a highly regioselective hydrolysis between the rings II and III to afford compound **5** in 75% isolated yield as its hydrochloride salt. We would like to note that the isolated material was found inadequate for biological tests, because the presence of some minute impurities including the starting material G418. Since these impurities could not completely taken off either by repeated re-crystallization or by flash column chromatography, we decided to perform two additional chemical steps: simultaneous conversion of all the amino groups into the corresponding azides (TfN_3, $CuSO_4$) to afford the compound **9**, which after Staudinger reaction generated the desired product **5** as a homogeneous material. For the synthesis of the pseudo-disaccharide **6**, however, the hydrolysis product of G418 (shown by dashed arrow in Scheme 1) was directly treated with $Zn(OAc)_2$ and Boc_2O to afford the selectively N-1-Boc-protected derivative (60% isolated yield), which after azidation (TfN_3, $CuSO_4$) gave the diazido derivative **10** in 88% isolated yield. Compound **10** was then converted to the corresponding N-1-AHB derivative **11** in three successive steps in the overall 70% yield for three steps. Thus, treatment of **10** with TFA gave the corresponding N-1 amine which after treatment with (*S*)-2-hydroxy-4-azidobutyric acid (HOBT, DCC, Et_3N) and incubation of the crude reaction mixture with methylamine afforded the selectively N-1-acylated product **11**. The Staudinger reaction then generated the desired product **6**.

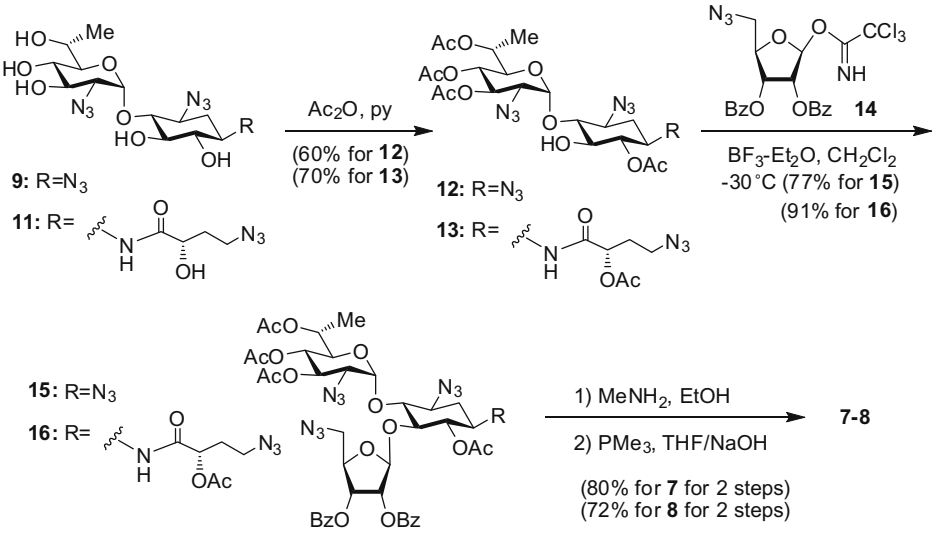
The synthesis of the pseudo-trisaccharides **7** and **8** was accomplished from the corresponding protected pseudo-disaccharide intermediates **9** and **11**, respectively, by using essentially the same chemical transformations as illustrated in Scheme 2. Regioselective acetylation of **9** and **11** with Ac_2O at low temperature gave the corresponding C5 acceptors **12** and **13** in 60% and 70% isolated yields, respectively. Our earlier studies with the paromamine (compound **3**)[25] and N-1-AHB-paromamine (compound **4**)[26] scaffolds have demonstrated that the corresponding C5 acceptors are highly unreactive in glycosylation reactions and only trichloroacetimidate donors gave satisfactory results. Taking this argument into account, for the glycosylation of **12** and **13** we used the trichloroacetimidate donor **14**,[25] which furnished the desired pseudo-trisaccharides **15** and **16** in 77% and 91% isolated yields, respectively, exclusively as beta-anomers at the newly generated glycosidic linkage. Two sequential deprotection steps: treatment with methylamine to remove all the ester protections, and the Staudinger reaction to convert azides to the corresponding amines, then gave the target compounds **7** and **8** in 80% and 72% isolated yields, respectively, for two steps. The structures of all new compounds (**5–8**) were confirmed by a combination of various 1D and 2D NMR techniques, including 2D ^1H–^{13}C HMQC and HMBC, 2D COSY, and 1D selective TOCSY experiments, along with mass spectral analysis.

2.2. Comparative in vitro stop codon suppression tests to evaluate the impact of (*R*)-6′-methyl group on pseudo-disaccharides (compounds 3–6) and pseudo-trisaccharides (compounds 1, 2, 7 and 8)

Initially, the influence of the (*R*)-6′-methyl group on readthrough potential was evaluated on pseudo-disaccharide structures by using a dual luciferase reporter assay system.[26,32,35] Briefly, the p2luc plasmid containing a TGA C nonsense mutation was transcribed and translated using the TNT reticulocyte lysate quick coupled transcription/translation system in the presence of varying concentrations of compounds **3–6**, and the stop codon suppression efficiency was calculated as previously reported[35] (Fig. 2A).



Scheme 1.



Scheme 2.

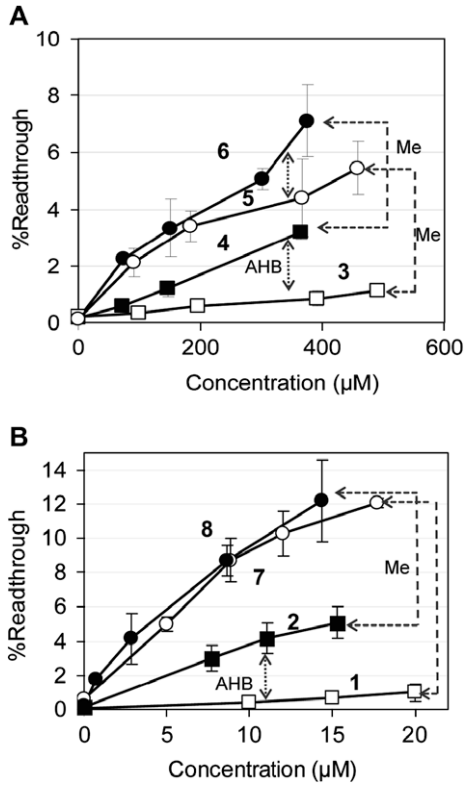


Figure 2. Levels of in vitro suppression of a TGA C nonsense mutation by (A) pseudo-disaccharide compounds **3** (□), **4** (■), **5** (○) and **6** (●), and (B) pseudo-trisaccharide compounds **1** (□), **2** (■), **7** (○) and **8** (●). The p2luc plasmid containing TGA C nonsense mutation in a polylinker between Renilla and Firefly luciferase genes[32] was transcribed and translated using the TNT reticulocyte lysate quick coupled transcription/translation system (Promega™). The luciferase activity was determined by using the Dual luciferase reporter assay system (Promega™) and the stop codon suppression efficiency was calculated as described previously.[35] The results are averages at least 2–3 independent experiments.

As seen from the data in Fig. 2A, installation of either N-1-AHB moiety (compound **4**) or (*R*)-6′-methyl group (compound **5**) on the paromamine scaffold (compound **3**) dramatically increases its in vitro readthrough activity. In addition, as depicted in the figure with dashed arrows, at all the concentrations tested, the impact of (*R*)-6′-methyl group is substantially higher versus that of N-1-AHB group; comparison of the gaps between **3**→**5** and **4**→**6** for the net effect of (*R*)-6′-methyl group, and the gaps between **3**→**4** and **5**→**6** for the net effect of N-1-AHB group. The pseudo-disaccharide **6** that contains both N-1-AHB and (*R*)-6′-methyl groups shows the highest suppression levels at all the concentrations tested. Whether the structure **6** is reached by **3**→**4**→**6** transition or by **3**→**5**→**6** transition, gradual increase of readthrough activity is observed, indicating about the additive influence of the two important structural elements, N-1-AHB and (*R*)-6′-methyl groups, on the observed readthrough activity. Nevertheless, the observed significantly higher suppression levels of the pseudo-disaccharides **5** and **6** compared to those of **3** and **4**, suggested that **5** and **6** can serve as improved scaffolds for the generation of new derivatives.

Our earlier structure–activity studies[25,26] demonstrated that the preservation of the pseudo-trisaccharide core structure of paromomycin (rings I–III) in compounds **1** and **2** is important for their efficient readthrough activity. Therefore, initially we focused on the construction and tests of the new pseudo-trisaccharides, compounds **7** and **8** that contain the 5-amino ribose as the ring III attached at position 5 of the new scaffolds **5** and **6**, respectively. Comparative in vitro suppression tests of the pseudo-trisaccharides **1**, **2**, **7**, and **8** were performed under the same experimental conditions as for the corresponding pseudo-disaccharides (compounds **3–6**, Fig. 2A), using p2luc plasmid containing a TGA C nonsense mutation, and the observed data are shown in Figure 2B.

Very similar trend in regards to the relative influence of the (*R*)-6′-methyl and N-1-AHB groups on the suppression efficiency was observed. Thus, the impact of (*R*)-6′-methyl group is substantially higher versus that of N-1-AHB group; at all concentrations tested, the gaps **1**→**7** and **2**→**8** (representing the differences only by the (*R*)-6′-methyl group) are significantly larger than those **1**→**2** and **7**→**8** (representing the differences only by the N-1-AHB group). While these observations were indeed very encouraging, we wanted to take them with certain precautions. This because the efficiency of aminoglycosides-induced readthrough is highly dependent on: (i) the identity of stop codon (UGA > UAG > UAA), (ii) the identity of the first nucleotide immediately downstream from the stop codon (C > U > A ⩾ G) and (iii) the local sequence context around the stop codon.[31,32] Therefore, in attempts to provide broader understanding on structure–activity relationship of the designed structures, we used a variety of constructs containing different sequence contexts around premature stop codons derived

from the *PCDH15*, *CFTR*, *Dystrophin* and *IDUA* genes that underlie USH1, CF, DMD and HS, respectively. The prevalent nonsense mutations of these diseases that we chose were: R3X and R245X for USH1, G542X and W1282X for CF, R3381X for DMD, and Q70X for HS. DNA fragments containing the nonsense mutation or the corresponding wild type codon, in their natural context, were cloned in frame between the Renilla and the Firefly luciferase genes of the p2luc vector as described previously by us.[26] The resulting six nonsense mutation-carrying plasmids, were transcribed and translated in the presence of varying concentrations of compounds **1**, **2**, **7**, **8** and gentamicin, and the stop codon suppression efficiency was calculated as previously reported[26,35] (Fig. 3).

From the observed data in Figure 3 it can be seen that the efficacy of readthrough is substantially different between different constructs and compounds tested, with no obvious dependence of readthrough effectiveness on the introduced type of modification on aminoglycoside. Nevertheless, in all mutations tested (except R3X, Fig. 3A) compound **8** induced the highest level of readthrough, followed by **2**, **7**, gentamicin, and **1**. The UGA C tetracodon sequence (R3X) showed the best translational readthrough than UGA A and UGA G, with the UAG C tetracodon least efficient, in agreement with earlier observations.[26,31,32] Inhibition of translation was monitored as the ratio of Renilla luciferase activity with and without the presence of aminoglycosides.[35] In all the constructs tested, the highest concentration of compound **8** resulted in approximately 50% reduction in overall translation, whereas the effects of **1**, **2**, **7**, and gentamicin on translation were significantly milder resulting in approximately 10–30% reduction in overall translation.

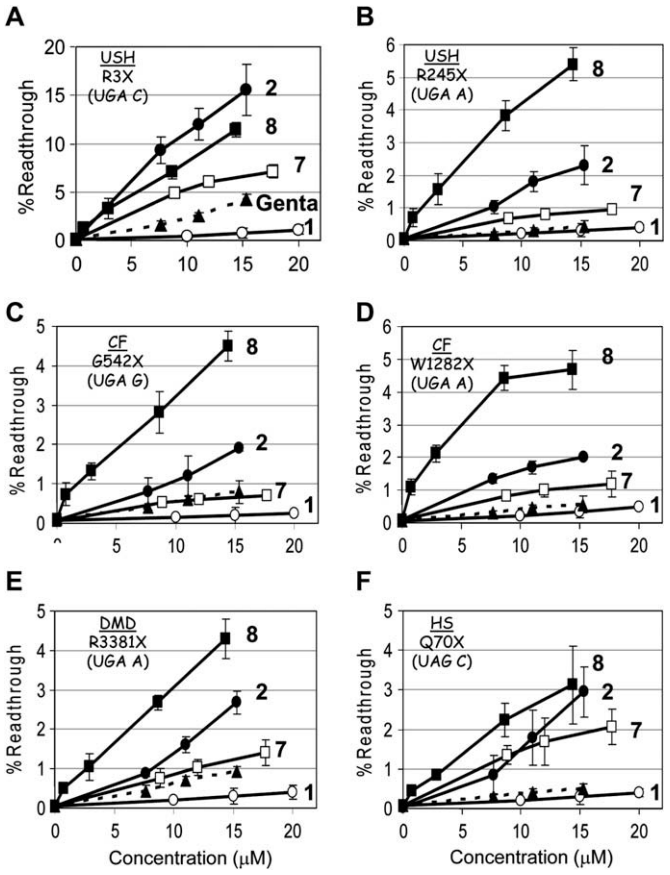


Figure 3. In vitro stop codon suppression levels induced by compounds **1** (○), **2** (●), **7** (□) **8** (■) and gentamicin (▲) in a series of nonsense mutation context constructs representing various genetic diseases (shown in parenthesis): (A) R3X (USH1), (B) (USH1), (C) G542X (CF), (D) W1282X (CF), (E) R3381X (DMD), and (F) Q70X (HS). DNA fragments were cloned between BamHI and SacI restriction sites of the p2luc vector and the obtained constructs were transcribed and translated using TNT quick coupled transcription/translation system.[26] The amount of the translated products was evaluated using the dual luciferase reporter assay system and the suppression level was calculated as described previously.[35] The results are averages of at least three independent experiments.

2.3. Comparative stop codon suppression tests in mammalian cell line

Cell line experiments were performed on three different dual luciferase reporter plasmids harboring the *PCDH15*-R3X nonsense mutation of USH1, the *IDUA*-Q70X nonsense mutation of HS, and the *CFTR*-W1282X nonsense mutation of CF. These reporter constructs were the same as we used above for the in vitro study, and have distinct advantage to control for differences in mRNA levels between normal and nonsense-containing sequences over those of single reporter or direct protein analysis, as previously noted.[32,35] The constructs were transfected into a human embryonic kidney cell line (HEK-293) and incubated with varying concentrations of **2**, **8**, G418 and gentamicin (Fig. 4). In a single case, *PCDH15*-R3X nonsense mutation, in addition to **2**, **8** and gentamicin, we also tested the compound **7** (Fig. 4A). For the clarity, the data with G418 are omitted in panels A–C and is only shown in panel D for *CFTR*-W1282X nonsense mutation, in comparison to that of compound **8**. Very similar preference of G418 over that of **8** was also observed in *PCDH15*-R3X and *IDUA*-Q70X nonsense mutations (data not shown). In order to ensure suitable cell viability for each of the tested compounds at the concentrations tested, we determined for each compound the half-maximal-lethal concentration value (LC_{50} values) in HEK-293cells (Table 1).

In all the mutations tested, the observed efficacy of aminoglycoside-induced readthrough was in the order G418 > **8** > **2** > gentamicin. This trend for **8** and **2** in Q70X and W1282X (Fig. 4B and C) was very similar to that observed for the suppression of the same stop mutations in vitro (Fig. 3). However, the observed higher readthrough potency of **8** over that of **2** in R3X was opposite to that of the same mutation in vitro. Interestingly, compound **7**, while exhibited significantly lower suppression activity than that of compound **2** in R3X mutation in vitro, in cell line both showed very similar activity. While these data may point to a better cell permeability of **7** and **8** over that of **2**, due to the presence of the (*R*)-6′-methyl group, it is clear that more experiments are needed to extract this issue satisfactorily. Nevertheless, the observed similar cell toxicity of the compounds **8** and **2** in HEK-293 cells (Table 1), along with substantially elevated suppression activity of **8** over that of **2** both in vitro and ex vivo in cultured cells, indicate that the compound **8** may represent a more superior choice than compound **2** in suppression therapy.

Furthermore, the comparative ex vivo suppression data in Fig. 4D, shows only about twofold preference of G418 over that of compound **8**, while the cell toxicity data in Table 1 indicate that G418 is about fivefold more toxic than **8**. Additional comparative toxicity tests, including nephrotoxicity and ototoxicity, the major drawbacks of aminoglycosides[24] are underway to validate the observed benefits of the compound **8** over that of G418 and gentamicin.

2.4. Compounds 7 and 8 inhibit prokaryotic protein translation with significantly lower potency and exhibit markedly reduced bactericidal activity and cell toxicity than those of gentamicin and G418

In our previous studies,[23,25,26] we have shown that compounds **1** and **2** are about 10-fold weaker inhibitors of prokaryotic translation than gentamicin and paromomycin and exhibit almost no

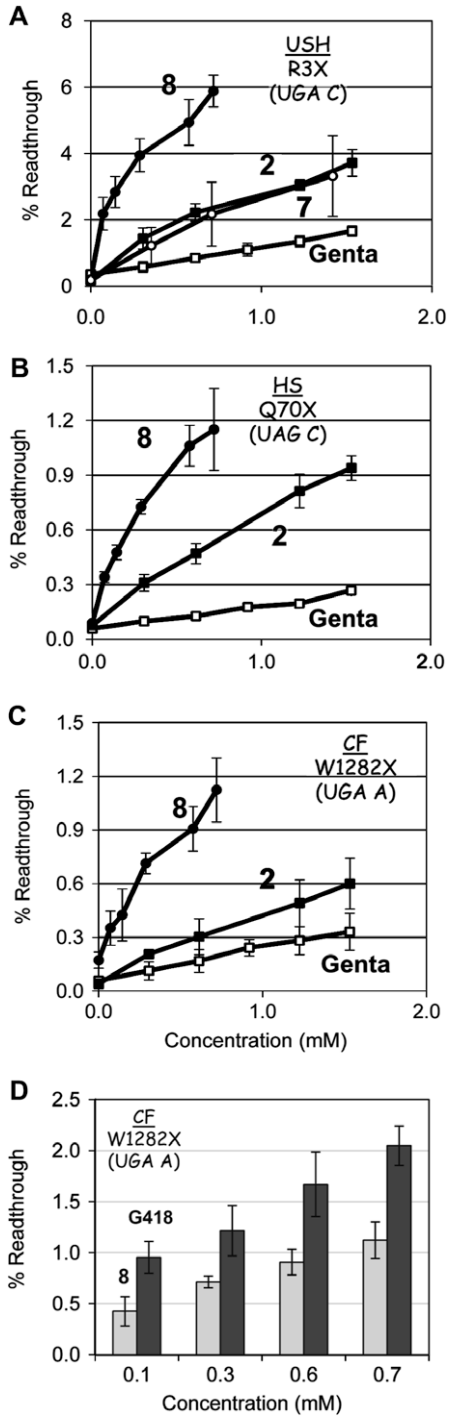


Figure 4. Ex vivo suppression of the (A) *PCDH15*-R3X, (B) *IDUA*-Q70X, and (C) *CFTR*-W1282X nonsense mutations by gentamicin (□), **2** (■), **7** (○), and **8** (●). The data with compound **7** are given only for *PCDH15*-R3X mutation in the panel A. Panel D represents comparative data between G418 and compd **8** for *CFTR*-W1282X mutation. The constructs of p2luc plasmid harboring the R3X, Q70X and W1282 mutations were transfected to HEK-293 cells and addition of the tested compounds was performed 6 h post transfection. Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega™). Stop codon readthrough was calculated as described previously.[35] The results are averages of at least three independent experiments.

bactericidal activity against both Gram-negative and Gram-positive bacteria. To assess whether the compounds **7** and **8** retain similar properties, we conducted comparative translation inhibition of **1**, **2**, **7**, **8**, G418 and gentamicin in a prokaryotic system, by using an in vitro luciferase assay (Table 1). The measured half-maximal inhibitory concentration (IC_{50}) values show that the efficacy with which both **7** and **8** inhibit the prokaryotic ribosome is significantly lower than that of gentamicin and G418. These data are in accordance with the observed antibacterial data of this set of compounds (Table 1). Thus, while gentamicin and G418 exhibit excellent antibacterial activities against both Gram-negative *Escherichia coli* (R477-100) and Gram-positive *Bacillus subtilis* (ATCC-6633), both **7** and **8** lack significant antibacterial activity. The observed data with **7** and **8** is similar to that observed for **1** and **2** and further support the previously reported correlation in aminoglycosides between prokaryotic antitranslational activity and MIC values: decreased inhibition of prokaryotic translation is associated with the decrease in antibacterial activity.[36]

We have previously reported that the compounds **1** and **2** exhibit significantly reduced cytotoxicity than those of gentamicin and paromomycin, as measured in three different kidney-derived cells.[26] To assess whether the compounds **7** and **8** retain similar properties, we determined comparative cell toxicity of **1**, **2**, **7**, **8**, G418 and gentamicin in HEK-293 cells (Table 1). The LC_{50} values obtained for **7** and **8** (25.8 and 7 mM) were similar to those for **1** and **2** (21.4 and 6.1 mM), and were 10- and 2.8-fold higher than that of the clinical aminoglycoside gentamicin (LC_{50} = 2.5 mM) Among all aminoglycosides tested the aminoglycoside antibiotic G418, which is known as one of the most cytotoxic aminoglycoside,[33] exhibited the lowest LC_{50} value (1.3 mM).

Comparison of the observed cell toxicity data in Table 1 with the readthrough activity data in Figure 3, demonstrates that the introduction of (*R*)-6′-methyl group either on compound **1** to give compound **7** (**1**→**7**), or on compound **2** to give compound **8** (**2**→**8**), not affects on the cytotoxicity (LC_{50} values of 21.4 and 25.8 mM for **1** and **7**, and 6.1 and 7.0 mM for **2** and **8**, respectively), while it greatly increases the observed stop codon suppression activity (**1** < **7** and **2** < **8**). This observation is one of the key outcomes of the present work. Furthermore, these data in conjunction with those observed for G418 and gentamicin indicate that the two unique structural elements of G418, the secondary (*R*)-6′-OH and the garosamine ring (ring III, Fig. 1), contribute differently to its biological performance. The secondary (*R*)-6′-OH most contributes to its elevated readthrough potency (G418 shows far higher suppression activity than gentamicin, Fig. 4C and D), while the garosamine ring donates the largest part to its high cytotoxicity (G418 shows only twofold lower LC_{50} than gentamicin in HEK-293 cells, Table 1). Gentamicin consists of the same garosamine ring at the same C6 position as G418 (Fig. 1), and essentially similar difference to that of HEK-293 cells was also observed in cytotoxicity assay in human fibroblast cells (3.8 mM and 2.0 mM for gentamicin and G418, respectively, see Section 5 and Fig. 2S in Supplementary data).

The impact of (*R*)-6′-methyl group on the elevated readthrough activities of **7**, **8** and G418 is further supported from the observed eukaryotic antitranslational data (Table 1). The efficacy with which G418 (IC_{50} = 2.0 μM) inhibits eukaryotic translation is greater than that of **8** (IC_{50} = 2.8 μM) and **7** (IC_{50} = 17.0 μM), a similar trend to that observed for readthrough activity: G418 > **8** > **7** (Fig. 4). Furthermore, the comparison of IC_{50} values of **7** and **8** to those of their parent structures **1** and **2** (IC_{50} values of 31 and 24 μM, respectively), reveals that **7** and **8** are 1.8-fold and 8.6-fold more specific to the eukaryotic ribosome than their parents **1** and **2**. Finally, although gentamicin contains 6′-NH_2, similar comparison between gentamicin (IC_{50} = 62 μM) and G418 (IC_{50} = 2.0 μM), reveals that G418 is 31-fold more specific than gentamicin. This collective data indicate that the observed impact of (*R*)-6′-methyl group on the elevated readthrough activities of **7**, **8** and G418 is associated with their increased specificity to the eukaryotic ribosome.

Table 1
Comparative cell toxicity, eukaryotic and prokaryotic translation inhibition, and antibacterial activity data of gentamicin, G418 and synthetic compounds **1**, **2**, **7** and **8**[a]

Aminoglycoside	Cell toxicity LC_{50}[b] (mM)	Translation inhibition[c]		Antibacterial activity MIC[d] (μM)	
		Eukaryotic system IC_{50} (μM)	Prokaryotic system IC_{50} (nM)	*E. coli* R477/100	*B. subtilis* ATCC6633
Gentamicin	2.5 ± 0.3	62 ± 9	28 ± 4	6	<0.75
G418	1.3 ± 0.1	2.0 ± 0.3	8.9 ± 1.5	9	<1.25
Compound **1**	21.4 ± 3.9	31 ± 4	460 ± 50	790	100
Compound **2**	6.1 ± 0.6	24 ± 1	160 ± 20	588	70
Compound **7**	25.8 ± 2.8	17.0 ± 0.6	1130 ± 120	680	42
Compound **8**	7.0 ± 0.6	2.8 ± 0.3	980 ± 70	556	70

[a] In all biological tests, all tested aminoglycosides were in their sulfate salt forms. The concentrations reported refer to that of the free amine form of each aminoglycoside.
[b] Aminoglycosides-induced cell toxicity was measured in human embryonic kidney cells, HEK-293, and calculated as a ratio between the number of living cells in cultures grown in the presence of the tested compound, versus cultures grown without compound. The half-maximal lethal concentration (LC_{50}) values were obtained from fitting concentration–response curves to the data of at least two independent experiments, using GraFit 5 software.
[c] Prokaryotic and eukaryotic translation inhibition was quantified in coupled transcription/translation assays as previously described by us.[23] The half-maximal concentration (IC_{50}) values were obtained from fitting concentration response curves to the data of at least two independent experiments, using GraFit 5 software.
[d] The MIC values were determined by using the double-microdilution method, with 384 μg/mL as starting concentration of each tested compound. All the experiments were performed in duplicates and analogous results were obtained in three different experiments.

3. Discussion

In the last several years, it was shown that besides their use as antibiotics, aminoglycosides could have therapeutic value in the treatment of human genetic disorders caused by nonsense mutations.[24,31] However, the most critical factor that largely limits the potential of aminoglycosides for suppression therapy is their high human toxicity.[24] Despite its high toxicity, the clinical drug gentamicin was/is frequently used for proof-of-concept experiments in various diseases models and in clinical trials, and no systematic study has been performed to tune aminoglycosides structures for better readthrough activity and low toxicity. Recently, we hypothesized that by separating the structural elements of aminoglycosides that induce readthrough from those that affect toxicity we might reach potent derivatives with improved readthrough activity and reduced toxicity.[24–26] By keeping this line of research, we systematically developed the lead compounds **1**[25] and **2**.[26] We initially identified the pseudo-disaccharide **3** (paromamine) as a minimal core structure exhibiting significant readthrough activity, and by attaching 5-amino ribose as a ring III spotted compound **1** as the first lead structure (Fig. 5). Continuing our efforts, we then 'borrowed' the pharmacophore AHB from the butirosin/amikacin and by installing it on compound **1** generated compound **2** as the second-generation lead structure.

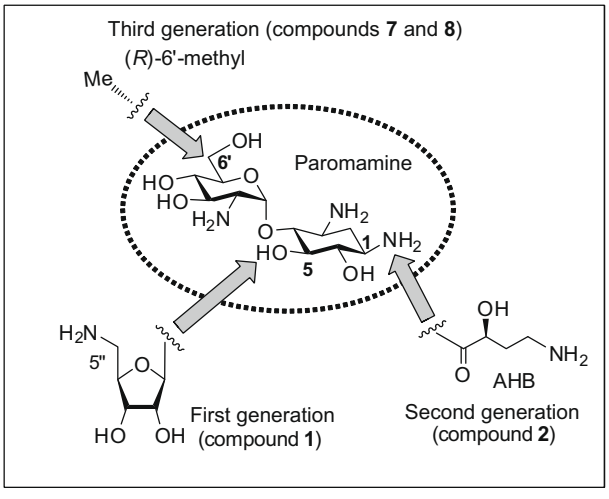


Figure 5. Chronological development of lead compounds on the paromamine (**3**) scaffold by systematic optimization of structure–activity–toxicity relationship as follow: first-generation compound **1**, by attaching 5-amino ribose at C5 position;[25] second-generation compound **2**, by further attaching AHB group at N-1 position of the compound **1**;[26] third-generation compounds **7** and **8**, by installing (*R*)-6′-Me on **1** and **2**, respectively (current work).

Further efforts within the above stream, which are described in this study lead us to the development of new lead compounds **7** and **8** (Fig. 5). Here we discovered a new pharmacophore, (*R*)-6′-methyl group, which we 'borrowed' from the natural aminoglycoside G418. The impact of (*R*)-6′-methyl group on the elevated readthrough activity was first demonstrated on pseudo-disaccharides **3–6** (Fig. 2). Comparative in vitro suppression tests clearly indicated that the installation of (*R*)-6′-methyl group either on **3** or **4** leads to substantially increased readthrough efficiency of the resulted structures **5** and **6** compared to those of their parent structures (**3** and **4**). Encouraged from the observed data we then installed (*R*)-6′-methyl group onto **1** and **2** to construct the pseudo-trisaccharides **7** and **8**, which were investigated in more details. The results of the current study show that both **7** and **8** exhibit substantially greater suppression activity and far lower toxicity than those of gentamicin. The evidence for the superior readthrough efficiency of **7** and **8** over that of gentamicin was demonstrated in vitro on six different DNA fragments derived from the mutant *PCDH15*, *CFTR*, *Dystrophin* and *IDUA* genes carrying nonsense mutations and representing the underlying causes for the genetic diseases USH1, CF, DMD and HS, respectively (Fig. 3), and ex vivo in cultured cell lines on three different DNA fragments that model the genetic diseases USH1, CF, and HS (Fig. 4). Furthermore, compound **8** also exhibits several-fold higher suppression activity than that of the previous lead compound **2**, while both (**8** and **2**) display similar cytotoxicity in HEK-293 cells (Table 1). Essentially the same trend was also observed in comparative study between the compounds **7** and **1**. The combined data suggests that installation of (*R*)-6′-methyl group on aminoglycoside structure to yield the (*R*)-6′-secondary alcohol on ring I, significantly increases suppression activity while has no significant influence on the cell toxicity of the resulted derivative.

Earlier studies have shown that aminoglycosides with a 6′-OH group on ring I (such as G418 and paromomycin, Fig. 1) are more effective than those with an amine at the same position.[31,37] One of the key differences between the prokaryotic and eukaryotic A-site is the nucleotide in the 1408 position: an adenine in the prokaryotes and a guanine in the eukaryotes. An A1408G mutation in various engineered bacteria leads to enhanced resistance towards aminoglycosides, but much higher levels of resistance are observed towards aminoglycosides with 6′-NH_2 than towards those with 6′-OH,[29,30] in agreement with the suppression results obtained in the mammalian system. The crystal structures of the bacterial A-site in complex with different aminoglycosides show that indeed the 6′ functional groups form key H-bonds with the A1408 base, and

models of the A1408G mutations based on these structures suggest that this mutation would prevent such interaction completely in 6′-NH_2 molecules.[30,38] A close check of these earlier data[29,30] revealed that although only paromomycin and G418 conferred significantly low resistance to the bacteria harboring A1408G mutation, there was noteworthy difference in the level of resistance between the paromomycin and G418: the relative resistances observed in different studies were 1–8-fold for G418 and 16–80-fold for paromomycin.[29,30,39] Based on this difference, we hypothesized that the presence of the secondary (*R*)-6′-OH group in G418 versus that of primary 6′-OH in paromomycin could, in principle, contribute to the observed significantly reduced resistance of G418 versus that of paromomycin.[40] This hypothesis is validated by our current data broadly showing that the compounds having secondary (*R*)-6′-OH group suppress premature stop codons more efficiently than their parent structures with the primary 6′-OH. The observed higher specificity to the eukaryotic ribosome, of the compounds containing (*R*)-6′-OH group than their parent structures with the primary 6′-OH, further supports this validation (Table 1). Finally, the results of this study also suggest that the structural element that most likely contributes to the extremely high readthrough activity of G418 is the (*R*)-6′-OH group, while the presence of C6-linked garosamine ring (ring III) primarily donates to its elevated cytotoxicity.

4. Summary and conclusions

In summary, the newly developed aminoglycosides compounds **7** and **8** exhibit both appreciably higher suppression efficiency and lower toxicity than those of gentamicin. This achievement has several significant implications. First, the results of this study further validates our design hypothesis that by separating the structural elements of aminoglycosides that induce readthrough from those that affect toxicity we can reach potent derivatives with improved readthrough activity and reduced toxicity.[24–26] Second, the observed continued inability of our previous leads, **1**[25] and **2**,[26] along with current **7** and **8**, to show significant antibacterial activity in conjunction with their decreased prokaryotic ribosome specificity, are striking and remain to be further investigated. Of greatest concern is whether these data are linked or not to the observed relatively reduced cell toxicity potential of **1**, **2**, **7** and **8**. This view is supported by the fact that the mammalian mitochondrial protein synthesis machinery is very similar to the prokaryotic machinery[41] and that the aminoglycoside-induced toxicity may, at least in part, be connected to drug-mediated dysfunction of the mitochondrial ribosome.[42,43] Third, the observed high impact of the (*R*)-6′-secondary alcohol on the elevated readthrough activity and specificity to the eukaryotic ribosome, along with no significant influence on the cell toxicity, support the feasibility of testing this novel pharmacophore for the generation of novel structures that may act as drug for the treatment of genetic diseases. Finally, the observed relatively low toxicity and high degree of potency of the new generation structures **7** and **8** in targeting all six different nonsense constructs underlying USH1, CF, DMD and HS, support the feasibility of testing these novel aminoglycosides in treating these diseases in animal and human subjects.

5. Experimental

5.1. General techniques

NMR spectra (including ^1H, ^{13}C, DEPT, 2D COSY, 1D TOCSY, HMQC, HMBC) were routinely recorded on a Bruker Avance™ 500 spectrometer, and chemical shifts reported (in ppm) are relative to internal Me_4Si ($\delta = 0.0$) with $CDCl_3$ as the solvent, and to HOD ($\delta = 4.63$) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker Avance™ 500 spectrometer at 125.8 MHz, and the chemical shifts reported (in ppm) relative to the residual solvent signal for $CDCl_3$ ($\delta = 77.00$), or to external sodium 2,2-dimethyl-2-silapentane sulfonate ($\delta = 0.0$) for D_2O as the solvent. Mass spectra analysis were obtained either on a Bruker Daltonix Apex 3 mass spectrometer under electron spray ionization (ESI), or by a TSQ-70B mass spectrometer (Finnigan Mat). Reactions were monitored by TLC on Silica Gel 60 F_{254} (0.25 mm, Merck), and spots were visualized by charring with a yellow solution containing $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ (120 g) and $(NH_4)_2Ce(NO_3)_6$ (5 g) in 10% H_2SO_4 (800 mL). Flash column chromatography was performed on Silica Gel 60 (70–230 mesh). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. G418 (geneticin), paromomycin and gentamicin were purchased from Sigma. All other chemicals and biochemicals, unless otherwise stated, were obtained from commercial sources. In all biological tests, all the tested aminoglycosides were in their sulfate salt forms [M_w (g/mol) of the sulfate salts were as follow: compd **3** (paromamine)—408.1, compd **4**—546.9, compd **5**—437.1, compd **6**—533.1, G418—692.7; gentamicin—653.2; compd **1** (NB30)—563.0; compd **2** (NB54)—652.8; compd **7** (NB74)—564.3; compd **8** (NB84)—685.9].

5.2. Synthetic part

5.2.1. 6′-(*R*)-Methyl-1,2′,3-triazido-paromamine (9)

The titled compound was prepared by direct acid promoted cleavage of G418. To a stirred anhydrous methanol (50 mL) was added acetyl chloride (25 mL) over 10 min at 0 °C. After about 15 min stirring, the commercial G418 sulfate (5.0 g, 7.20 mmol) was added and the reaction was heated to reflux. Propagation of the reaction was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15], which indicated completion after 16 h. The reaction mixture was evaporated under vacuum to dryness and then purified by flash chromatography. The column was washed with the mixture of CH_2Cl_2/MeOH (1:1, 200 mL), followed by MeOH (500 mL). The product was eluted with the mixture of 5% NH_4OH in MeOH/H_2O [(25% NH_4OH solution in H_2O):(15% H_2O):(80% MeOH)] to yield compound **5** as a powder (1.8 gr, 75%). NMR (500 MHz, D_2O, pD 3.5): '*Ring I*': δ_H 1.10 (d, 3H, $J = 6.5$ Hz, CH_3-6), 3.34 (dd, 1H, $J_1 = J_2 = 10.0$ Hz, H-4), 3.36 (dd, 1H, $J_1 = 4.0$, $J_2 = 10.5$ Hz, H-2), 3.80 (dd, 1H, $J_1 = 9.0$, $J_2 = 10.0$ Hz, H-3), 3.78–3.82 (m, 1H, H-5), 4.11–4.15 (m, 1H, H-6), 5.50 (d, 1H, $J = 4.0$ Hz, H-1); '*Ring II*': δ_H 1.79 (ddd, 1H, $J_1 = J_2 = J_3 = 12.5$ Hz, H-2ax), 2.41 (dt, 1H, $J_1 = 4.0$, $J_2 = 12.5$ Hz, H-2eq), 3.21–3.26 (m, 1H, H-1), 3.48–3.52 (m, 1H, H-3), 3.51 (dd, 1H, $J_1 = J_2 = 9.5$ Hz, H-6), 3.57 (dd, 1H, $J_1 = J_2 = 9.0$ Hz, H-5), 3.77 (dd, 1H, $J_1 = J_2 = 9.5$ Hz, H-4). ^{13}C (NMR 125 MHz, D_2O): δ_C 16.2 (CH_3-6′), 30.0 (C-2), 50.9, 51.4, 55.9, 67.0, 71.2, 71.9, 74.0, 76.2, 77.0, 83.3, 99.3 (C-1′); MALDI TOFMS calcd for $C_{13}H_{27}N_3O_7$ ($[M+H]^+$) *m*/*e* 338.2; measured *m*/*e* 338.2)

We would like to note that the product from the above step contained about 1% of the starting G418 along with other impurities (~1%, originally exist in the starting material). These impurities could not be completely removed by repeated column purifications. Therefore, the product from the above step (1.8 g, 5.30 mmol) was converted to the corresponding 1,3,2′-triazido derivative by following the published procedure,[44] using Tf_2O (8.0 mL, 48.0 mmol) and NaN_3 (3.13 g, 48.0 mol). The reaction progress was monitored by TLC (EtOAc/MeOH 95:5), which indicated completion after 8 h. The resulted crude was purified by flash chromatography (EtOAc/hexane 95:5) to yield compound **9** (1.98 g, 90% yield). NMR (500 MHz, MeOD): '*Ring I*': δ_H 1.24 (d, 3H, $J = 6.0$ Hz, CH_3-6), 3.07 (dd, 1H, $J_1 = 4.0$, $J_2 = 10.0$ Hz, H-2), 3.36 (dd, 1H, $J_1 = 8.5$, $J_2 = 9.5$, H-4), 3.92 (dd, 1H, $J_1 = J_2 = 9.5$ Hz, H-3), 3.92–

3.95 (m, 1H, 1H, H-5), 4.01–4.04 (m, 1H, H-6), 5.72 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.37 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.22 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.24 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-6), 3.38–3.43 (m, 1H, H-1), 3.48–3.52 (m, 1H, H-3), 3.38–3.43 (m, 1H, H-5), 3.47–3.53 (m, 1H, H-4). ^{13}C (NMR 125 MHz, MeOD): δ_C 16.8 (CH_3-6′), 32.2 (C-2), 59.9, 60.6, 63.5, 68.1, 71.2, 73.0, 74.1, 76.8, 76.8, 78.8, 97.5 (C-1′); ESI calcd for $C_{13}H_{20}N_9O_7$ ([M−H]$^-$) m/e 414.2; measured m/e 414.1).

5.2.2. 6′-(*R*)-Methyl-paromamine (5)

Compound **9** (56 mg, 0.14 mmol) was dissolved in a mixture of THF (3.0 mL) and aqueous NaOH (1 mM, 3.0 mL). This mixture was stirred at room temperature for 10 min, after which PMe_3 (1 M solution in THF, 1.1 mL, 1.10 mmol) was added. The reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15], which indicated completion after 1 h. The reaction mixture was purified by flash chromatography on a short column of silica gel. The column was washed with the following solvents: THF (100 mL), CH_2Cl_2 (100 mL), EtOH (50 mL), and MeOH (100 mL). The product was then eluted with the mixture of 20% $MeNH_2$ solution (33% solution in EtOH) in 80% MeOH. Fractions containing the product were combined and evaporated under vacuum. The residue was re-dissolved in a small volume of water and evaporated again (2–3 repeats) to afford the free amine form of compound **5**. The analytically pure product was obtained by passing the above product through a short column of Amberlite CG50 (NH_4^+ form). First, the column was washed with water, then the product was eluted with a mixture of 10% NH_4OH in water to yield compound **5** (32.0 mg, 70%). For the storage and biological tests, compound **5** was converted to its sulfate salt form as follow. The free base form was dissolved in water, the pH was adjusted to 6.7 with H_2SO_4 (0.1 N) and lyophilized to afford the sulfate salt of **5** as a white foamy solid. ^{1}H NMR (500 MHz, D_2O, pD 3.5) '*Ring I*': δ_H 1.10 (d, 3H, J = 6.5 Hz, CH_3-6), 3.34 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-4), 3.36 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz, H-2), 3.80 (dd, 1H, J_1 = 9.0, J_2 = 10.0 Hz, H-3), 3.78–3.82 (m, 1H, H-5), 4.11–4.15 (m, 1H, H-6), 5.50 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.79 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.41 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.21–3.26 (m, 1H, H-1), 3.48–3.52 (m, 1H, H-3), 3.51 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-6), 3.57 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-5), 3.77 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-4). ^{13}C (NMR 125 MHz, D_2O): δ_C 16.2 (CH_3-6′), 30.0 (C-2), 50.9, 51.4, 55.9, 67.0, 71.2, 71.9, 74.0, 76.2, 77.0, 83.3, 99.3 (C-1′); MALDI TOFMS calcd for $C_{13}H_{27}N_3O_7$ ([M+H]$^+$) m/e 338.2; measured m/e 338.2).

5.2.3. 2′,3-Diazido-1-*N*-[(*tert*-butoxy)carbonyl]-6′-(*R*)-methyl-paromamine (10)

$Zn(OAc)_2$ (6.0 g, 27.00 mmol) was added to a stirred solution of compound **5** in its free base form (7.0 g, 21.00 mmol) in H_2O (30 mL) and DMF (150 mL), and the mixture was stirred for 12 h at room temperature. A solution of di-*tert*-butyldicarbonate (6.0 g, 27.00 mmol) in DMF (20 mL) was added to the reaction mixture over a time period of 30 min and the mixture was stirred for an additional 24 h. The reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15]. The reaction mixture was diluted with MeOH (250 mL) and loaded onto 50 mm × 300 mm ion-exchange column (Amberlite CG50, H^+ form). The column was first washed extensively (about 10 column volumes) with a mixture MeOH/H_2O (50:50), followed by elution with the mixture of MeOH/H_2O/NH_4OH (25% solution in water) (20:3:2) to afford the desired N-1-Boc derivative in 60% yield (5.5 g). ^{1}H NMR (500 MHz, D_2O): '*Ring I*': δ_H 1.12 (d, 3H, J = 6.5 Hz, CH_3-6), 2.73 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz, H-2), 3.25 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-4), 3.49 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-3), 3.76 (dd, 1H, J_1 = 2.0, J_2 = 10.0 Hz, H-5), 4.03–4.08 (m, 1H, H-6), 5.14 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.20 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 1.89–1.96 (m, 1H, H-2eq), 2.75–2.83 (m, 1H, H-3), 3.18 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-4), 3.22 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-6), 3.32–3.39 (m, 1H, H-1), 3.47 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-5); the additional peak in the spectrum was identified as follow: δ_H 1.34 (s, 9H, Boc). ^{13}C NMR (125 MHz, D_2O): δ_C 16.7 (CH_3-6′), 29.4 (Boc, 3C), 36.2 (C-2), 51.0, 57.0, 67.9, 71.6, 72.9, 75.5, 76.4, 76.5, 77.6, 88.7, 102.6 (C-1′), 159.7 (Boc, CO). MALDI TOFMS calcd for $C_{18}H_{35}N_3O_9$ ([M+H]$^+$ m/e 438.2; measured m/e 438.2).

The product from the above step (5.5 g, 12.60 mmol) was converted to the corresponding 3,2′-diazido derivative by following the published procedure,[44] using Tf_2O (20 mL, 120 mmol) and NaN_3 (10 g, 153 mmol). The reaction progress was monitored by TLC (EtOAc/MeOH 9:1), which indicated completion after 8 h. The crude product was purified by flash chromatography (EtOAc/hexane 85:15) to yield the title compound **10** (5.4 g, 88% yield). ^{1}H NMR (500 MHz, $CDCl_3$): '*Ring I*': δ_H 1.03 (d, 3H, J = 6.5 Hz, CH_3-6), 2.99 (dd, 1H, J_1 = 4.0, J_2 = 10.0 Hz, H-2), 3.20 (dd, 1H, J_1 = 9.0, J_2 = 9.5, H-4), 3.59–3.62 (m, 1H, H-5), 3.72 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-3), 3.76–3.80 (m, 1H, H-6), 5.33 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.18 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.08–2.16 (m, H-2eq), 2.98 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-6), 3.20–3.28 (m, 2H, H-1, H-3), 3.20 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-4), 3.31 (dd, 1H, $J_1 = J_2$ = 9.0 Hz, H-5); the additional peaks in the spectrum were identified as follow: δ_H 1.23 (s, 9H, Boc). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.4 (CH_3-6′), 27.7 (Boc, 3C), 32.9 (C-2), 48.8 (C-1), 59.4, 62.9, 68.6, 71.1, 73.0, 73.2, 74.2, 76.6, 79.9, 97.3 (C-1′), 159.3 (Boc, CO). MALDI TOFMS calcd for $C_{18}H_{32}N_7O_9$ ([M+H]$^+$) m/e 490.2; measured m/e 490.3).

5.2.4. 2′,3-Diazido-1-*N*-[(*S*)-4-azido-2-hydroxybutanoyl]-6′-(*R*)-methyl-paromamine (11)

Compound **10** (1.3 g, 2.76 mmol) was dissolved in a mixture of trifluoroacetic acid (12 mL) and dichloromethane (30 mL). The reaction progress was monitored by TLC (CH_2Cl_2/MeOH 8:2), which indicated completion after 1 h. The reaction mixture was concentrated to dryness under reduced pressure. The resulting crude was dissolved in a mixture of Et_3N (10 mL) and DMF (10 mL) and cooled to −30 °C. In a separate flask, (*S*)-2-hyroxy-4-azidobutyric acid (2.0 g, 15.78 mmol) was dissolved in anhydrous DMF (30 mL) and cooled to 0 °C. To the cold solution, DCC (3.6 g, 17.23 mmol) and HOBT (2.4 g, 17.48 mmol) were added and the resulted mixture was stirred at 0 °C for about 1 h. This mixture was carefully added by syringe to the cold solution of the amine at −30 °C. The reaction was stirred at −30 °C for 1 h and then allowed to warm to room temperature for an additional 1 h. The mixture was then treated with a solution of $MeNH_2$ (33% solution in EtOH, 30 mL) and the reaction progress was monitored by TLC (CH_2Cl_2/MeOH 8:2). After completion of the reaction (about 8 h) the mixture was concentrated and purified by flash chromatography (MeOH/CH_2Cl_2 1:9) to provide the titled compound **11** (1.0 g, 70% yield). ^{1}H NMR (500 MHz, MeOD): '*Ring I*': δ_H 1.24 (d, 3H, J = 5.0 Hz, CH_3-6), 3.20 (dd, 1H, J_1 = 4.5, J_2 = 10.0 Hz, H-2), 3.42 (dd, 1H, J = 9.5 Hz, H-4), 3.81–3.85 (m, 1H, H-5), 3.94 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-3), 3.95–3.99 (m, 1H, H-6), 5.53 (d, 1H, J = 3.0 Hz, H-1); '*Ring II*': δ_H 1.45 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.24–2.29 (m, 1H, H-2eq), 3.34 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-6), 3.41 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-5), 3.42–3.48 (m, 1H, H-3), 3.74 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-4), 3.73–3.78 (m, 1H, H-1); the additional peaks in the spectrum were identified as follows: δ 1.81–1.89 (m, 1H), 2.05–2.10 (m, 1H), 3.42–3.46 (m, 2H), 4.14 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz). ^{13}C (NMR 125 MHz, MeOD): δ_C 17.6 (CH_3-6′), 32.3 (C-2), 32.8, 47.2, 50.5, 50.7, 59.4, 68.7, 68.8, 71.1, 73.0, 73.4, 73.8, 76.5, 80.0, 97.3 (C-1′), 174.7 (CO). MALDI TOFMS calcd for $C_{17}H_{29}N_{10}O_9$ ([M+H]$^+$) m/e 516.2; measured m/e 516.2).

5.2.5. 2′,3-Diamino-1-*N*-[(*S*)-4-amino-2-hydroxybutanoyl]-6′-(*R*)-methyl-paromamine (6)

The titled compound was prepared as was described for the preparation of compound **5** with the following quantities: compound **11** (70.0 mg, 0.14 mmol), THF (3 mL), NaOH (1 mM, 3.0 mL), PMe_3 (1 M solution in THF, 2.00 mL, 2.00 mmol) to yield compound **6** as a free amine form (39 mg, 65%). ^{1}H NMR (500 MHz, D_2O, pD 6.5): '*Ring I*': δ_H 1.14 (d, 3H, J = 7.0 Hz, CH_3-6), 3.18 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz, H-2), 3.33 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-4), 3.71 (dd, 1H, J_1 = 10.5, J_2 = 10.0 Hz, H-3), 3.82–3.85 (m, 1H, H-5), 4.11–4.15 (m, 1H, H-6), 5.39 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.73 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.09–2.13 (m, 1H, H-2eq), 3.25–3.30 (m, 1H, H-3), 3.48 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-6), 3.58 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-5), 3.61 (dd, 1H, $J_1 = J_2$ = 9.5 Hz, H-4), 3.78–3.84 (m, 1H, H-1); the additional peaks in the spectrum were identified as follows: δ 1.90–1.97 (m, 1H), 2.07–2.10 (m, 1H), 3.06–3.14 (m, 2H), 4.25 (dd, 1H, J_1 = 4.0, J_2 = 7.5 Hz). ^{13}C (NMR 125 MHz, D_2O): δ_C 14.5 (CH_3-6′), 30.8 (C-2), 31.2, 36.5, 49.4, 50.9, 54.5, 65.5, 69.5, 70.4, 70.9, 73.5, 75.0, 75.3, 83.6, 98.5 (C-1′), 175.5 (CO). MALDI TOFMS calcd for $C_{17}H_{34}N_4O_9K$ ([M+K]$^+$) m/e 477.2; measured m/e 477.1).

5.2.6. 3′,4′,6′,6-Tetra-*O*-actate-2′,3-diazido-1-*N*-[(*S*)-4-azido-2-*O*-acetate-butanoyl]-6′-(*R*)-methyl-paromamine (12)

Compound **9** (900 mg, 2.20 mmol) was dissolved in dry pyridine (10 mL), cooled at −12 °C and then acetic anhydride (4.5 equiv, 1.0 mL, 10.0 mmol) was added. The temperature was kept at −12 °C and the reaction progress was monitored by TLC (EtOAc/hexane 6:4), which indicated completion after 16 h. The reaction mixture was diluted with EtOAc (100 mL) and extracted with aqueous solutions of $NaHCO_3$, HCl (2%), saturated aqueous $NaHCO_3$, and brine. The combined organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (EtOAc/hexane 3:7) to afford **12** (840 mg, 60% yield). ^{1}H NMR (500 MHz, $CDCl_3$): '*Ring I*': δ_H 1.24 (d, 3H, J = 6.0 Hz, CH_3-6), 3.56 (dd, 1H, J_1 = 3.5, J_2 = 10.5 Hz, H-2), 4.31 (dd, 1H, J_1 = 2.0, J_2 = 10.5 Hz, H-5), 4.92–4.98 (m, 1H, H-6), 4.96 (dd, 1H, J_1 = 10.0, J_2 = 9.0 Hz, H-4), 5.43 (d, 1H, J = 4.0 Hz, H-1), 5.45 (dd, 1H, J_1 = 9.0, J_2 = 10.0 Hz, H-3); '*Ring II*': δ_H 1.56 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.36 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.34–3.40 (m, 1H, H-1), 3.50–3.55 (m, 1H, H-3), 3.47 (dd, 1H, J_1 = 9.0, J_2 = 10.0 Hz, H-4), 3.67 (dd, 1H, J_1 = 9.0, J_2 = 9.5 Hz, H-5), 4.89 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-6); the additional peaks in the spectrum were identified as follow: 2.07 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.15 (s, 3H, Ac). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 14.0 (CH_3-6′), 20.6–21.0 (Ac, 4C), 32.0 (C-2), 57.9, 58.4, 61.5, 68.7, 69.1, 70.8, 71.5, 74.5, 75.1, 82.4, 98.4 (C-1′), 169.8, 170.0, 170.2, 170.5. MALDI TOFMS calcd for $C_{21}H_{29}N_9O_{11}$ Na ([M+Na]$^+$) m/e 606.2; measured m/e 606.3)

5.2.7. 3′,4′,6′,6-Tetra-*O*-actate-2′,3-diazido-1-*N*-[(*S*)-4-azido-2-*O*-acetate-butanoyl]-6′-(*R*)-methyl-paromamine (13)

The titled compound was prepared as was described for the preparation of compound **12** with the following quantities: compound **11** (1.0 g, 1.94 mmol), pyridine (10 mL) and acetic anhydride (5.5 equiv, 1.0 mL, 10.60 mmol). Yield: 1.1 g (70%). ^{1}H NMR (500 MHz, $CDCl_3$): '*Ring I*': δ_H 1.25 (d, 3H, J = 7.0 Hz, CH_3-6), 3.62 (dd, 1H, J_1 = 3.5, J_2 = 10.5 Hz, H-2), 4.34 (dd, 1H, J_1 = 2.0, J_2 = 10.5 Hz, H-5), 4.96–5.00 (m, 1H, H-6), 4.98 (dd, 1H, J_1 = 10.5, J_2 = 9.0 Hz, H-4), 5.35 (d, 1H, J = 4.5 Hz, H-1), 5.50 (dd, 1H, J_1 = 9.0, J_2 = 10.5 Hz, H-3); '*Ring II*': δ_H 1.47 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.50 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.39–3.45 (m, 1H, H-3), 3.38–3.43 (m, 1H, H-4), 3.77 (dd, 1H, J_1 = 9.0, J_2 = 9.5 Hz, H-5), 3.95–4.03 (m, 1H, H-1), 4.80 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-6), 6.62 (d, 1H, J = 7.5 Hz, NH); the additional peaks in the spectrum were identified as follow: 2.05–2.13 (m, 2H), 2.06 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.15 (s, 3H, Ac), 2.19 (s, 3H, Ac), 3.36–3.40 (m, 2H), 5.14 (dd, 1H, J_1 = 5.0, J_2 = 6.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 14.0 (CH_3-6′), 20.6–21.0 (Ac, 5C), 30.5, 32.6 (C-2), 47.0, 48.3, 58.3, 61.7, 68.8, 69.0, 70.9, 70.9, 71.4, 74.0, 74.1, 83.6, 98.5 (C-1′), 169.1, 169.8, 170.0, 170.0, 170.1, 172.5. MALDI TOFMS calcd for $C_{27}H_{38}N_{10}O_{14}$ K ([M+K]$^+$) m/e 765.2; measured m/e 765.2).

5.2.8. 5-*O*-(5-Azido-2,3-*O*-dibenzoyl-5-deoxy-β-D-ribofuranose)-3′,4′,6′,6-tetra-*O*-actate-1,2′,3-triazido-6′-(*R*)-methyl-paromamine (15)

To powdered, flame-dried 4 Å molecular sieves (1.5 g) was added anhydrous CH_2Cl_2 (12 mL), followed by the addition of acceptor **12** (600 mg, 1.03 mmol) and donor **14**[25] (2.2 g, 4.06 mmol). The mixture was stirred for 10 min at room temperature and was then cooled to −30 °C. A catalytic amount of BF_3–Et_2O (100 μL) was added and the mixture was continued to stir at −30 °C. The reaction progress was monitored by TLC (EtOAc/hexane 35:65), which indicated completion after 15 min. The reaction was diluted with EtOAc and filtered through Celite. After thorough washing of the Celite with EtOAc, the washes were combined and extracted with saturated aqueous $NaHCO_3$, brine, dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography to yield **15** (750 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$): '*Ring I*': δ_H 1.27 (d, 3H, J = 6.0 Hz, CH_3-6), 3.50 (dd, 1H, J_1 = 4.0, J_2 = 11.0 Hz, H-2), 4.45 (dd, 1H, J_1 = 0.7, J_2 = 10.5 Hz, H-5), 4.97 (dd, 1H, J_1 = 9.5, J_2 = 10.0 Hz, H-4), 4.98–5.02 (m, 1H, H-6), 5.44 (dd, 1H, J = 10.0 Hz, H-3), 5.94 (d, 1H, J = 4.0 Hz, H-1); '*Ring II*': δ_H 1.51 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.39 (dt, 1H, J_1 = 4.0, J_2 = 13.0 Hz, H-2eq), 3.48–3.58 (m, 2H, H-1, H-3), 3.76 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-4), 3.91 (dd, 1H, J = 10.0 Hz, H-5), 5.03 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-6); '*Ring III*': δ_H 3.58 (dd, 1H, J_1 = 6.0, J_2 = 13.0 Hz, H-5′), 3.64 (dd, 1H, J_1 = 3.0, J_2 = 13.0 Hz, H-5), 4.53–4.56 (m, 1H, H-4), 5.42 (dd, 1H, J_1 = 5.0, J_2 = 10.0 Hz, H-3), 5.62 (d, 1H, J = 5.0 Hz, H-2), 5.64 (s, 1H, H-1); the additional peaks in the spectrum were identified as follow: 2.05–2.09 (m, 12H, Ac), 7.36 (t, 2H, J = 8.0, Bz), 7.42 (t, 2H, J = 8.0 Hz, Bz), 7.53–7.55 (m, 1H, Bz), 7.57–7.60 (m, 1H, Bz), 7.88 (dd, 2H, J_1 = 1.0, J_2 = 7.5 Hz, Bz), 7.95 (dd, 2H, J_1 = 1.0, J_2 = 7.5 Hz, Bz). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 13.3 (CH_3-6′), 20.6–21.2 (Ac, 4C), 32.1 (C-2), 53.1 (C-5″), 58.3, 58.9, 61.6, 68.5, 69.0, 70.2, 70.6, 72.0, 74.0, 74.5, 76.8, 80.0, 80.8, 96.2 (C-1′), 107.6 (C-1″), 128.5 (Bz, 2C), 128.6 (Bz, 2C), 129.6 (Bz, 2C), 129.7 (Bz, 2C), 133.7 (Bz, 2C), 165.2 (Bz, CO), 165.4 (Bz, CO), 169.9–170.2 (4Ac, CO). MALDI TOFMS calcd for $C_{40}H_{44}N_{12}O_{16}$ K ([M+K]$^+$) m/e 971.3; measured m/e 971.3).

5.2.9. 5-*O*-(5-Azido-2,3-*O*-dibenzoyl-5-deoxy-β-D-ribofuranose)-3′,4′,6′,6-tetra-*O*-actate-2′,3-diazido-1-*N*-[(*S*)-4-azido-2-*O*-acetate-butanoyl]-6′-(*R*)-methyl-paromamine (16)

The titled compound was prepared as was described for the preparation of compound **15** with the following quantities: compound **13** (400 mg, 0.55 mmol), donor **14** (1.50 g, 2.84 mmol). Yield: 550 mg (91%). ^{1}H NMR (500 MHz, $CDCl_3$): '*Ring I*': δ_H 1.26 (d, 3H, J = 6.0 Hz, CH_3-6), 3.46 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz, H-2), 4.46 (dd, 1H, J_1 = 2.0, J_2 = 10.5 Hz, H-5), 4.96–5.01 (m, 1H, H-6), 5.00 (dd, 1H, $J_1 = J_2$ = 10.0 Hz, H-4), 5.44 (dd, 1H, J_1 = 9.5, J_2 = 10.0 Hz, H-3), 5.92 (d, 1H, J = 3.5 Hz, H-1); '*Ring II*': δ_H 1.58 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.50 (dt, 1H, J_1 = 4.0, J_2 = 13.0 Hz, H-2eq), 3.59–3.64 (m, 1H, H-3), 3.74 (dd, 1H, J_1 = 9.0, J_2 = 8.0 Hz, H-4), 3.98–4.03 (m, 1H, H-1), 4.00 (dd, 1H, J_1 = 8.5, J_2 = 9.0 Hz, H-5), 4.92 (dd, 1H, J_1 = 9.5, J_2 = 10.0 Hz, H-6), 6.64 (d, 1H, J = 7.5 Hz, NH); '*Ring III*': δ_H 3.60–3.68 (m, 2H, H-5), 4.52–4.57 (m, 1H, H-4), 5.50 (dd, 1H, J_1 = 5.0, J_2 = 7.0 Hz, H-3), 5.63 (dd, 1H, J = 5.0 Hz, H-2), 5.67 (s, 1H, H-1); the additional peaks in the spectrum were identified as follow: 2.03–2.15 (m, 2H), 2.08 (s, 3H, Ac), 2.09 (s, 6H, 2Ac), 2.19 (s, 3H, Ac), 2.23 (s, 3H, Ac), 3.35

(dd, 2H J_1 = 7.0, J_2 = 6.5 Hz), 5.16 (dd, 1H, J_1 = 5.0, J_2 = 7.0 Hz), 7.34 (dd, 2H, J_1 = J_2= 7.5 Hz, Bz), 7.43 (dd, 2H, J_1 = J_2= 7.5 Hz, Bz), 7.54 (dd, 1H, J_1 = J_2 = 7.5 Hz, Bz), 7.59 (dd, 1H, J_1 = J_2 = 7.5 Hz, Bz), 7.86 (dd, 2H, J_1 = 1.0, J_2 = 8.5 Hz, Bz), 7.95 (dd, 2H, J_1 = 1.0, J_2 = 8.5 Hz, Bz). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 15.4 (CH_3-6′), 22.5–23.0 (Ac, 5C), 26.5, 32.3 (C-2), 48.9, 50.3, 54.5 (C-5″), 60.5, 63.3, 70.4, 70.8, 72.1, 72.5 72.7, 73.3 75.3, 76.5, 79.0, 81.9, 81.9, 98.2 (C-1′), 109.2 (C-1″), 130.3 (Bz, 4C), 130.4 (Bz, 2C), 131.5 (Bz, 4C), 135.5 (Bz, 2C), 167.0 (Bz, CO), 167.1 (Bz, CO), 170.8 (AHB, CO), 171.6–174.2 (5Ac, CO). MALDI TOFMS calcd for $C_{46}H_{53}N_{13}O_{19}$ Na ([M+Na]$^+$) *m*/*e* 1114.3; measured *m*/*e* 1114.5).

5.2.10. 6′-(*R*)-Methyl-5-*O*-(5-amino-5-deoxy-β-D-ribofuranose)-2′,1,3-triamino-paromamine (7)

Compound **15** (750 mg, 0.79 mmol) was treated with a solution of $MeNH_2$ (33% solution in EtOH, 30 mL) at room temperature and the reaction progress was monitored by TLC (EtOAc/MeOH 8:2), which indicated completion after 8 h. The reaction mixture was evaporated to dryness and was subjected to a Staudinger reaction as described above for the preparation of compound **5** under following conditions: THF (6.0 mL), NaOH (1 mM, 5.0 mL), PMe_3 (7.0 mL, 7.0 mmol) to yield **7** as a free amine form (295 mg, 80% yield). ^{1}H NMR (500 MHz, D_2O, pD 3.5) '*Ring I*': δ_H 1.09 (d, 3H, *J* = 6.5 Hz, CH_3-6), 3.32–3.36 (m, 1H, H-4), 3.36 (dd, 1H, J_1 = 4.0, J_2 = 9.0 Hz, H-2), 3.70 (dd, 1H, J_1 = 2.5, J_2 = 10.0 Hz, 1H, H-5), 3.83 (dd, 1H, J_1 = 9.0, J_2 = 10.0 Hz, H-3), 4.09–4.15 (m, 1H, H-6), 5.64 (d, 1H, *J* = 4.0 Hz, H-1); '*Ring II*': δ_H 1.78 (ddd, 1H, J_1 = J_2 = J_3 = 12.5 Hz, H-2ax), 2.37 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.22–3.28 (m, 1H, H-1), 3.45–3.51 (m, 1H, H-3), 3.65 (dd, 1H, J_1 = J_2 = 9.5 Hz, H-6), 3.88 (dd, 1H, J_1 = J_2 = 9.5 Hz, H-5), 3.99 (dd, 1H, J_1 = J_2 = 9.5 Hz, H-4); '*Ring III*': δ_H 3.11 (dd, 1H, J_1 = 8.0, J_2 = 13.5 Hz, H-5), 3.25–3.29 (m, 1H, H-5′), 3.99–4.05 (m, 1H, H-4), 4.08–4.11 (m, 1H, H-3), 4.14 (d, 1H, *J* = 5.0, H-2), 5.26 (d, 1H, *J* = 1.0, H-1). ^{13}C NMR (125 MHz, D_2O): δ_C 14.6 (CH_3-6′), 27.7 (C-2), 41.5 (C-5″), 49.2, 49.7, 53.6, 65.4, 69.2, 69.9, 70.8, 71.4, 74.4, 75.4, 77.5, 78.2, 82.0, 95.0 (C-1′), 108.5 (C-1″). MALDI TOFMS calcd for $C_{18}H_{37}N_4O_{10}$ ([M+H]$^+$) *m*/*e* 469.2; measured *m*/*e* 469.2).

5.2.11. 6′-(*R*)-Methyl-5-*O*-(5-amino-5-deoxy-β-D-ribofuranose)-2′,3-diamino-1-*N*-[(*S*)-4-amino-2-hydroxybutanoyl]paromamine

Compound **16** (520 mg, 0.48 mmol) was treated with a solution of $MeNH_2$ (33% solution in EtOH, 20 mL) and the reaction progress was monitored by TLC (EtOAc/MeOH 8:2), which indicated completion after 12 h. The reaction mixture was evaporated to dryness and was directly subjected to a Staudinger reaction as was described above for the preparation of compound **3** with the following quantities: THF (3.0 mL), NaOH (1 mM, 3.0 mL), PMe_3 (4.00 mL, 4.00 mmol) to afford **8** as a free amine form (195 mg, 72% yield). ^{1}H NMR (500 MHz, D_2O, pD 3.3): '*Ring I*': δ_H 1.16 (d, 3H, *J* = 6.5 Hz, CH_3-6), 3.40 (dd, 1H, J_1 = 9.0, J_2 = 10.0 Hz, H-4), 3.42 (dd, 1H, J_1 = 4.0, J_2 = 10.5 Hz, H-2), 3.76 (dd, 1H, J_1 = 2.5, J_2 = 10.0 Hz, H-5), 3.90 (dd, 1H, J_1 = J_2 = 10.0 Hz, H-3), 4.17–4.19 (m, 1H, H-6), 5.70 (d, 1H, *J* = 4.0 Hz, H-1); '*Ring II*': δ_H 1.72 (ddd, 1H, J_1 = J_2 = J_3 = 12.5 Hz, H-2ax), 2.15 (dt, 1H, J_1 = 4.0, J_2 = 12.5 Hz, H-2eq), 3.46–3.52 (m, 1H, H-3), 3.64 (dd, 1H, J_1 = 9.5, J_2 = 10.0 Hz, H-6), 3.83–3.88 (m, 1H, H-1), 3.89 (dd, 1H, J_1 = J_2 = 9.0 Hz, H-5), 3.99 (dd, 1H, J_1 = J_2 = 9.5 Hz, H-4); '*Ring III*': δ_H 3.16 (dd, 1H, J_1 = 7.5, J_2 = 13.5 Hz, H-5), 3.32 (dd, 1H, J_1 = 3.5, J_2 = 13.5 Hz, H-5′), 4.02–4.08 (m, 1H, H-4), 4.13 (dd, 1H, J_1 = 5.0, J_2 = 7.5 Hz, H-3), 4.16–4.19 (m, 1H, H-2), 5.31 (s, 1H, H-1); the additional peaks in the spectrum were identified as follow: 1.88–1.97 (m, 1H), 2.05–2.12 (m, 1H), 3.02–3.12 (m, 2H), 4.25 (dd, 1H, J_1 = 4.0, J_2 = 8.0 Hz). ^{13}C NMR (125 MHz, D_2O) : δ_C 16.5 (CH_3-6′), 31.5 (C-2), 32.6, 38.4, 43.3 (C-5″), 50.4, 51.6, 55.6, 67.3, 71.1, 71.4, 71.8, 72.7, 74.4, 76.3, 77.3, 79.7, 79.9, 84.8, 96.8 (C-1′), 110.4 (C-1″), 177.4. MALDI TOFMS calcd for $C_{22}H_{43}N_5O_{12}K$ ([M+K]$^+$) *m*/*e* 608.3; measured *m*/*e* 608.3).

5.3. Dual luciferase readthrough assays

DNA fragments derived from *PCDH15*, *CFTR*, *Dystrophin*, and *IDUA* cDNAs, including the tested nonsense mutation or the corresponding wild type codon and four to six upstream and downstream flanking codons, were created and inserted into the polylinker of the p2luc plasmid, as previously described by us.[26] For the in vitro readthrough assays, the obtained plasmids or the original p2luc plasmid, with addition of the tested aminoglycosides, were transcribed and translated using the TNT reticulocyte lysate quick coupled transcription/translation system (Promega™). Luciferase activity was determined after 90 min of incubation at 30 °C, using the Dual luciferase reporter assay system (Promega™). For the ex vivo readthrough assays, the constructs harboring the R3X, Q70X and W1282 mutations were transfected to HEK-293 cells with Lipofectamine 2000 (Invitrogen) and addition of the tested compounds was performed 6 h post transfection. The cells were harvested following 16 h incubation with the aminoglycosides. Stop codon readthrough was calculated as previously described.[35] The experimental details for all the readthrough experiments were identical to our recently reported procedures for the in vitro and ex vivo suppression tests of aminoglycosides variants.[25,26]

5.4. Protein translation inhibition tests

Prokaryotic in vitro translation inhibition by the different aminoglycosides was quantified in coupled transcription/translation assays[36] by using *E. coli* S30 extract for circular DNA with the pBESTluc plasmid (Promega), according to manufacturer's protocol. Translation reactions (25 μL) that contained variable concentrations of the tested aminoglycoside were incubated at 37 °C for 60 min, cooled on ice for 5 min, and diluted with a dilution reagent (tris-phosphate buffer (25 mM, pH 7.8), DTT (2 mM), 1,2-diaminocyclohexanetetraacetate (2 mM), glycerol (10%), Triton X-100 (1%) and BSA (1 mg mL^{-1})) into 96-well plates. Eukaryotic in vitro translation inhibition was quantified by use of TNT® T7 Quick Coupled Transcription/Translation System with a luciferase T7 control DNA plasmid (Promega), according to the manufacturer protocol. Translation reactions (25 μL) containing variable concentrations of the tested aminoglycoside were incubated at 30 °C for 60 min, cooled on ice for 5 min, diluted with the dilution reagent and transferred into 96-well plates. In both prokaryotic and eukaryotic systems the luminescence was measured immediately after the addition of the luciferase assay reagent (50 μL; Promega), and the light emission was recorded with an FLx800 Fluorescence Microplate Reader (Biotek). The half-maximal inhibition concentration (IC_{50}) values were obtained from fitting concentration–response curves to the data of at least two independent experiments by using Grafit 5 software,[45] and are presented in Table 1 and Figure 1S (see Supplementary data).

5.5. Antibacterial activity and human cell toxicity tests

Comparative antibacterial activities were determined in two representative strains of Gram-negative (*E. coli* R477–100) and Gram-positive (*B. subtilis* ATCC-6633) bacteria, by measuring the MIC values using the double-microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS)[46] with starting concentration of 384 μg/mL of the tested compound. All the experiments were performed in triplicates and analogous results were obtained in three different experiments.

For the cytotoxicity assays, HEK-293 cells were grown over night in 96-well plates (5000 cells/well) in DMEM medium containing 10% FBS, 1% penicillin/streptomycin and 1% glutamine (90 μL; Biological Industries) at 37 °C and 5% CO_2. The medium

was then changed with the medium without penicillin/streptomycin (90 μL), different concentrations of the tested aminoglycosides were added (10 μL) and the cells were incubated for 48 h. A cell proliferation assay (XTT based colorimetric assay, Biological Industries) was performed by using the 3-h incubation protocol, according to manufacturer's instructions. O.D. (optical density) was then read by using an Elisa plate reader. Cell viability was calculated as the ratio between the numbers of living cells in cultures grown in the presence of the tested compounds, versus cultures grown under the identical protocol but without the tested compound. Primary human dermal fibroblasts (HDF) were isolated from adult skin and cultivated as previously described.[47] Cytotoxicity assays in HDF cells were performed as described above for HEK-293 cells, except the following modification: the HDK cells were incubated with aminoglycosides for 72 h. The half-maximal lethal concentration (LC_{50}) values were obtained from fitting concentration–response curves to the data of at least two independent experiments, using GraFit 5 software[45] and are presented in Table 1 and Figure 2S (see Supplementary data).

Acknowledgments

We thank John F. Atkins (University of Utah) for providing the p2luc plasmid. This work was supported by research grants from the US-Israel Binational Science Foundation (Grant No. 2006/301) and by Mitchel Fund (Grant No. 2012386). I.N. acknowledges the Irwin and Joan Jacobs Fellowship; D.G. acknowledges the Miriam and Aaron Gutwirth Memorial Fellowship; V.B. acknowledges the financial support by the Center of Absorption in Science, the Ministry of Immigration Absorption and the Ministry of Science and Technology, Israel (Kamea Program).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.060.

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