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Redesign of aminoglycosides for treatment of human genetic diseases caused by premature stop mutations

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Abstract—A series of new derivatives of the clinically used aminoglycoside antibiotic paromomycin were designed, synthesized, and their ability to read-through premature stop codon mutations was examined in both in vitro translation system and ex vivo mammalian cultured cells. One of these structures, a pseudo-trisaccharide derivative, showed notably higher stop codon read-through activity in cultured cells compared to those of paromomycin and gentamicin.

A large number of human genetic disorders result from nonsense mutations, single point alterations in the DNA, where one of the three stop codons (TAA, TAG or TGA) replaces an amino acid-coding codon, leading to premature termination of the translation and eventually to truncated, nonfunctional proteins. Currently, hundreds of such mutations are known,[1] and for many of those diseases there is presently no effective treatment.

Aminoglycosides are highly potent, broad-spectrum antibiotics that exert their antibacterial therapeutic effect by selectively binding to the decoding aminoacyl site (A-site) of the bacterial 16S rRNA, and interfering with translational fidelity during protein synthesis.[2] Interestingly, in the last several years, numerous experiments performed either in vitro in mammalian translation systems, cultured cell lines, or animal models confirmed the ability of certain types of aminoglycoside antibiotics (Fig. 1) to induce mammalian ribosomes to read-through stop codon mutations via insertion of a random amino acid by a nearcognate tRNA. This unique activity was shown to generate full-length functional proteins in several genetic disorders.[3] Furthermore, clinical trials with cystic fibrosis patients clearly showed that aminoglycosides can suppress premature stop mutations in affected patients.[4] However, unfortunately, this great excitement which continues over the last two decades was largely hampered because of the following reasons. First and foremost, aminoglycosides are highly toxic to mammals (nephrotoxicity and ototoxicity), and the use of subtoxic doses in clinical trials resulted with the reduced read-through efficiency probably insufficient for

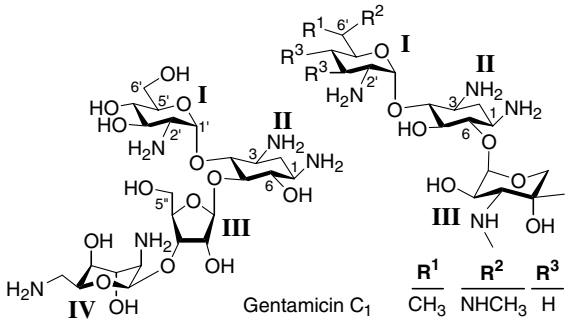

Figure 1. Aminoglycosides with stop codon read-through activity.

Keywords: Aminoglycosides; Cystic fibrosis; Drug design; Stop mutation suppression; Usher syndrome.
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successful therapy. Second, unlike recent insights into our understanding of how aminoglycosides might induce deleterious misreading of the genetic code in prokaryote cells,[5] the molecular mechanism of aminoglycoside-induced nonsense mutation suppression in mammalian cells remains to be established. Third, to date, nearly all suppression experiments have been performed with clinical, commercially available aminoglycosides,[6] and no efforts have been made to optimize their activity as stop codon read-through inducers. Clearly, a systematic search for new structures with improved termination suppression activity and lower toxicity is required to extrapolate the approach to the point where it can actually help patients.

For this purpose, as an initial trial, we have modified paromomycin and prepared a series of derivatives, **1**–**9** (Fig. 2). In selecting paromomycin as the modification target we have taken into consideration the following points. First, although to date there are not enough data to answer the question why some aminoglycosides induce termination suppression, while others do not, from the available data, it turns out that aminoglycosides with a C6′ hydroxyl group on ring I (such as G-418 and paromomycin, Fig. 1) are generally more effective than those with the amine functionality at the same position.[6] Second, paromomycin is the least toxic among the aminoglycosides that show considerably high suppression activity.[7] Based on these data we reasoned that by dissecting paromomycin structure (via selectively removing one or two sugar rings) we could identify a minimal structural motif with significant suppression activity, which then can be used as a scaffold for the construction of diverse structures with improved termination suppression and probably with lower toxicity.

Structures **2**–**7** preserve rings I and II of paromomycin (paromamine **1** moiety) as the minimal structural motif of paromomycin that binds to the mammalian ribosome and has significant suppression activity (vide infra). The extended sugar ring (ring III) in each structure is either the plain ribose (structures **2**, **4**, and **6**) or 5-amino ribose (structures **3**, **5**, and **7**) attached at C5, C6, and C3′ of **1**, respectively. The rational in selecting the ribose as a third sugar ring in **4** and **6** was to retain the identity of this sugar as in the parent paromomycin, and in parallel to explore another hitherto unknown areas of the mammalian A-site rRNA with possible location of new modes of binding. Since at physiological pH aminoglycosides are highly charged and their interaction with rRNA is mainly determined by electrostatic interaction,[8] we reasoned that by adding additional aminosugar to the paromamine **1** moiety, superior binding to mammalian rRNA and probably better suppression activity will result. Therefore, we selected 5-amino ribose as a third sugar ring and prepared the new generation of pseudotrisaccharides **3**, **5**, and **7** with the expectation that they will possibly function better as read-through inducers than the parallel structures containing the plain ribose ring (structures **2**, **4**, and **6**). Similar arguments, the direct addition of an extra amino group to the paromamine **1** moiety and its dimerization, served as a basis for the preparation of compounds **8** and **9**, respectively. Enhanced RNA binding by using dimerized aminoglycosides[9] and amino-aminoglycosides[10] supports these designs.

All the designed structures **1**–**9** were synthesized according to the general strategy (Fig. 2) that involves direct Lewis acid promoted cleavage of paromomycin into the pseudo-disaccharide **1**, which is then used as a common starting material for the preparation of all the designed structures. For the construction of pseudotrisaccharides **2**–**7**, we employed the appropriately protected three different paromamine acceptors, **11**–**13**, which selectively expose C5, C6, and C3′ hydroxyl groups of the paromamine moiety, respectively, for glycosidation reactions. These acceptor molecules were readily accessible from paromamine **1** as illustrated in Scheme 1. Simultaneous conversion of all the amino groups of **1** into the corresponding azides was done by treatment with TfN_3 to afford **10**. Regioselective

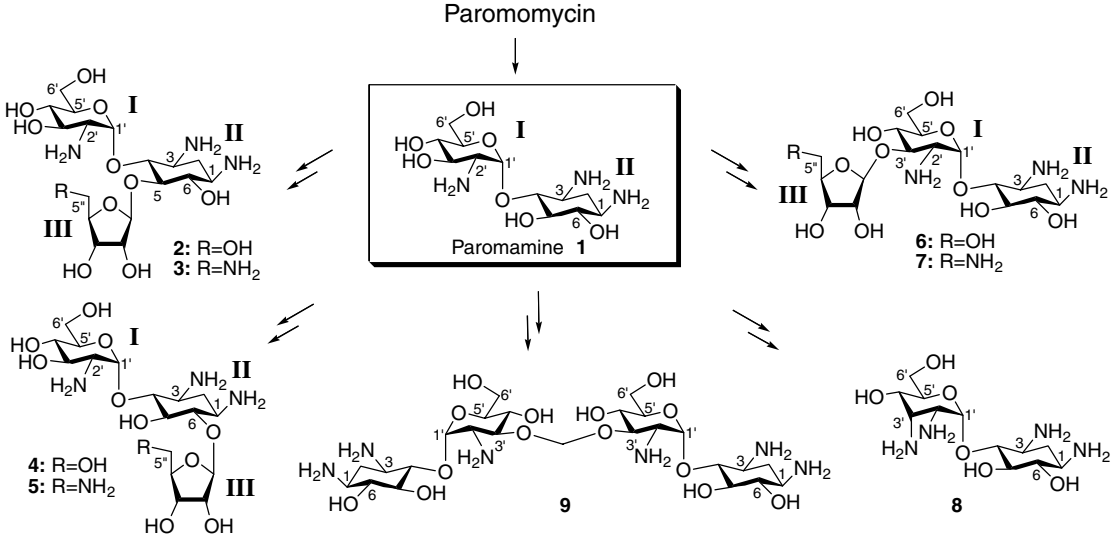
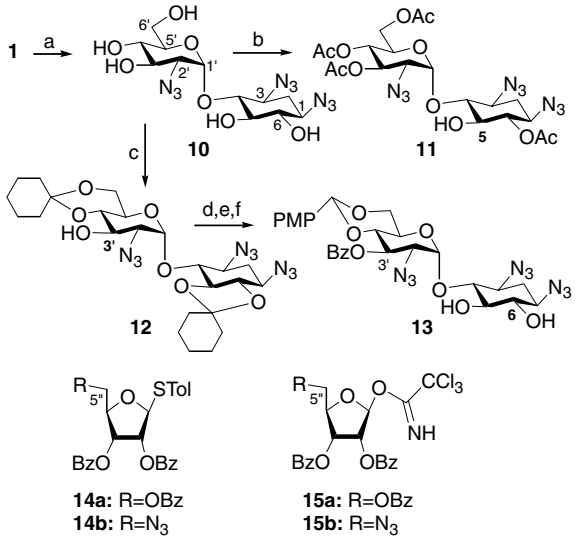


Figure 2. Structures of paromamine **1** and its synthetic derivatives **2**–**9**.

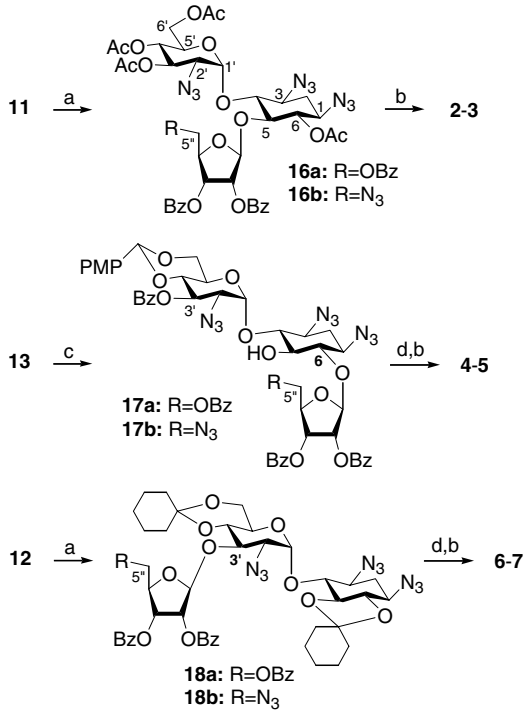


Scheme 1. Reagents and conditions: (a) TfN_3, Et_3N, $CuSO_4$, in CH_2Cl_2/MeOH/H_2O 3:10:3, (90%); (b) Ac_2O (4.2 equiv), pyridine, −6 °C, (65%); (c) cyclohexanone dimethyl ketal, CSA, DMF, 110 °C, (67%); (d) BzCl, pyridine; (e) TFA/H_2O 5:3, THF, 40 °C, (89% for the two steps); (f) anisaldehyde-dimethylacetal, CSA, DMF, 50 °C, (84%). Tf, trifluoromethanesulfonyl; CSA, camphor sulfonic acid; DMF, dimethylformamide; Bz, benzoyl; TFA, trifluoroacetic acid.

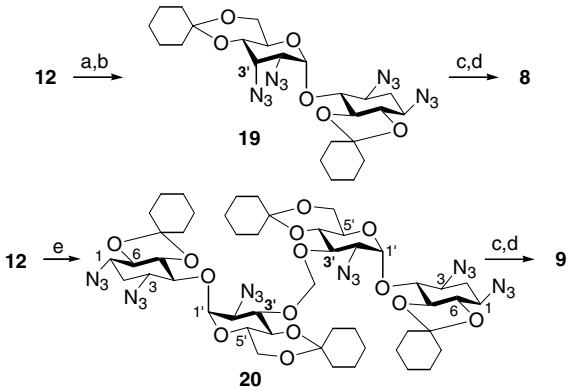
acetylation of **10** with acetic anhydride at low temperature gave acceptor **11**.[11] In another pathway, treatment of **10** with cyclohexanone dimethyl ketal gave the second acceptor, **12**, in which all functional groups, except the C3′-OH, are protected. Benzoylation of **12** was followed by acid hydrolysis and benzylidene acetal formation steps to afford the third acceptor **13**.

The acceptors **11**–**13** were separately subjected to glycosidation reactions with two sets of glycosyl donors, **14a**–**b** and **15a**–**b**,[12] to furnish the designed protected derivatives **16**–**18** in 68–95% yields (Scheme 2). These protected compounds were then subjected to either two step or three step deprotection to furnish the final pseudo-trisaccharide products **2**–**7** with excellent purity and isolated yields.

Ring I in the pseudo-disaccharide **8** has D-*allo* configuration and was constructed from the paromamine **1** by selectively inverting the configuration at C3′ (Scheme 3). Triflation of the C3′-OH in **12** was followed by nucleophilic displacement with azide to afford the corresponding *cis*-diazide **19**. Hydrolysis of the cyclohexylidene ketals with aqueous acetic acid, followed by the Staudinger reaction, provided the designed pseudo-disaccharide **8**. Treatment of the same acceptor **12** with CH_2Br_2 in the presence of NaH gave the protected dimer **20**, which after the similar deprotection as in the case of **19**, afforded the designed dimer **9**. The structures of **1**–**9** were confirmed by a combination of various techniques, including HMQC, HMBC, 2D COSY, and 1D TOCSY NMR, along with mass spectral analysis. For the biological assays, all compounds (**1**–**9**) were used in their sulfate salt forms, obtained by titration of the free base forms with diluted sulfuric acid.



Scheme 2. Reagents and conditions: (a) **15a** or **15b** BF_3–Et_2O (cat), CH_2Cl_2, 4 Å molecular sieves, **11** → **16a** (85%), **11** → **16b** (71%), **12** → **18a** (95%), **12** → **18b** (93%); (b) i—$MeNH_2$ (33% soln in EtOH), ii—PMe_3 (1 M in THF), NaOH 0.1 M, THF, rt; **16a** → **2** (84%), **16b** → **3** (91%), **17a** → **4** (for two steps 75%), **17b** → **5** (44%), **18a** → **6** (84%),**18b** → **7** (75%); (c) **14a** or **14b** NIS, TfOH (cat), CH_2Cl_2, 4 Å molecular sieves,**13** → **17a** (68%), **13** → **17b** (76%); (d) AcOH/H_2O 6:1,THF 50 °C for **17a**, TFA/H_2O 3:2, THF, 60 °C for **17b** (52%), AcOH/H_2O 10:3, 1,4-dioxane, 70 °C for **18a** (75%), TFA/H_2O 5:1, THF, 50 °C for **18b** (82%). PMP, *p*-methoxyphenyl, NIS, *N*-iodosuccinimide.



Scheme 3. Reagents and conditions: (a) Tf_2O, pyridine, (92%); (b) NaN_3, DMF, HMPA, (72%) (c) for **19** AcOH/H_2O 8:1, 1,4-dioxane, 75 °C, (60%), for **20** TFA/H_2O 5:6, THF, 60 °C, (90%); (d) i—$MeNH_2$ (33% soln in EtOH), ii—PMe_3 (1 M in THF), NaOH 0.1 M, THF, rt; **19** → **8** (76%), **20** → **9** (81%), (e) CH_2Br_2, NaH, DMF/HMPA 2:1, 4 Å molecular sieves, (82%). HMPA, hexamethylphosphoramide.

Initially, derivatives **1**–**9** were tested for their ability to suppress a nonsense mutation in vitro, using a reporter construct carrying an R3X nonsense mutation (a premature UGA C stop codon) of the *PCDH15* gene (Table 1, Fig. 3).[13,14] Each compound was assayed at several

Table 1. Maximal in vitro suppression and translation levels of the R3X mutation, along with the MIC values measured for the designed structures **1–9**

Compound[a]	Concn (μg mL^{-1})	Supp. level[b] (%)	Trans. level[b] (%)	MIC[c] (μg mL^{-1})	
				E. coli	*B. Subtilis*
Paromomycin	40	49 ± 6	40 ± 13	12	8
Gentamicin	30	49 ± 4	40 ± 9	4	<0.5
Paromamine (**1**)	80	6.2 ± 0.2	74 ± 15	512	128
2	80	1.3 ± 0.1	100 ± 10	256	64
3	80	21 ± 3	72 ± 6	>512	48
4	80	1.5 ± 0.1	74 ± 7	256	96
5	80	4.4 ± 2	75 ± 9	>512	192
6	160	<1	82 ± 8	>512	>512
7	80	2.9 ± 2	98 ± 10	192	48
8	80	2.0 ± 2	71 ± 7	192	48
9	80	<1	22 ± 2	96	48

[a] All the designed structures tested (**1–9**), along with paromomycin and gentamicin, were in their sulfate salt forms.

[b] The determination of the suppression and translation levels were performed as in the footnote of Figure 3. The results are averages of at least three independent experiments.

[c] The MIC values were determined using the double-microdilution method, with two different starting concentrations of the tested compounds (384 and 512 μg/mL). The bacterial strains used were *Escherichia coli* (ATCC 25922), and *Bacillus Subtilis* (ATCC 6633). All the experiments were performed in triplicates and analogous results were obtained in two to four different experiments.

different concentrations and the concentrations at which maximal suppression levels were observed are given in Table 1.

As seen from the data in Table 1, removal of either one ring (ring IV) or two rings (rings III and IV) in paromomycin dramatically decreases its maximal in vitro read-through activity from 49% suppression to 1.3% (compound **2**) and 6.2% (compound **1**), respectively. The substantially higher suppression level of **1** (6.2%) compared to that of the pseudo-trisaccharide fragment **2** (1.3%) implies that paromamine **1** represents the minimal structural motif of paromomycin that has significant suppression activity. Connection of the plain ribose ring to the paromamine scaffold either at C6 (compound **4**) or C3′ position (compound **6**), along with the addition of one extra amine (**8**) or paromamine dimerization (**9**), gave lower suppression levels than that of paromamine itself.

The most important results, however, were observed when instead of plain ribose, the 5-amino ribose was connected at different positions. The observed suppression levels of all these derivatives (compounds **3**, **5**, and **7**) are higher than the parallel structures containing plain ribose ring at the same location (**2**, **4**, and **6**, respectively). Compound **3**, in which the ribosamine is attached to C5 of the paromamine structure, shows the highest suppression level among the new derivatives. The maximal suppression level of **3** (21%) was obtained at 80 μg mL^{-1}, a concentration which is much higher than the concentrations required for the maximal activities of both paromomycin and gentamicin (Table 1, Fig. 3). Nevertheless, the fact that compound **3** still retained translation level of 72% at 80 μg mL^{-1} is also a very important result, as discussed below.

In the series of pseudo-trisaccharides **3**, **5**, and **7**, a particular influence of the position of the ribosamine ring is observed: C5 (**3**) ≫ C6 (**5**) > C3′ (**7**), suggesting that the preservation of the pseudo-trisaccharide core structure of the parent paromomycin (rings I-III) in **3** is important for efficient read-through activity. The suppression data of **2–7** show that in the series of pseudo-trisaccharides, an increased number of amino groups in each pair leads to improved read-through activity, in agreement with the reports about enhanced RNA binding of aminoglycoside derivatives with extra amino groups.[9,10] However, the data obtained with **8** and **9** indicate that merely increasing the number of amino groups on the paromamine scaffold does not always lead to an increase in read-through activity. Nevertheless, the observed 13-fold higher suppression level of derivative **3** compared to that of the corresponding ribose derivative **2,** and over 3-fold better activity compared to that of paromamine **1**, suggest that the presence of C5″-NH_2 in **3** is responsible for its elevated read-through activity.

To further evaluate the read-through potential of compound **3**, its activity was assayed in cultured mammalian cells using a dual luciferase reporter system,[16] and compared to the activities of paromomycin and gentamicin (Fig. 4). At all concentrations tested, the activity of **3** is superior to those of paromomycin and gentamicin. In addition, gentamicin (Fig. 1), which is currently the only clinically relevant aminoglycoside shown to have the ability to suppress nonsense mutations in patients, is less efficient than either paromomycin or **3**. The improved read-through efficiency of compound **3** relative to those of paromomycin and gentamicin in the whole-cell system (in contrast to the in vitro results) indicates that the effective concentration of compound **3** inside the cell and near the ribosome is higher than the effective concentrations of paromomycin and gentamicin. This could be an outcome of better penetration and improved uptake of **3** by the cells, resulting from its smaller size compared to paromomycin and gentamicin. In addition, since compound **3** has one less positive charge (+4) than either paromomycin (+5) or gentamicin

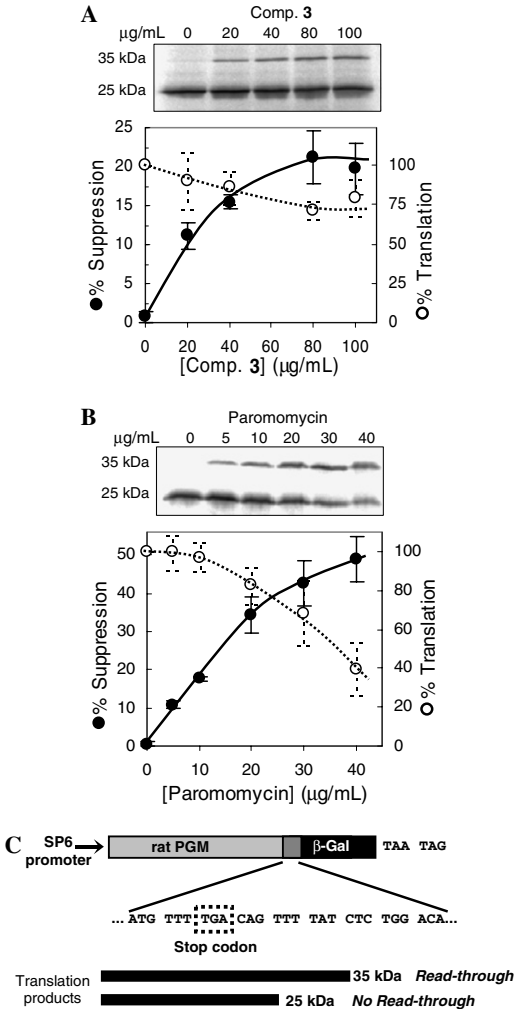


Figure 3. Levels of in vitro suppression of a nonsense mutation (•) and overall translation (○) by compound **3** (A) and by paromomycin (B). Panel C describes the reporter system used for the in vitro translation assays. A plasmid-based reporter construct contains a TGA C nonsense mutation between a 25-kDa polypeptide encoding open reading frame (ORF) and a 10-kDa polypeptide encoding ORF, such that efficient translation termination at the stop codon results in the production of a 25-kDa polypeptide, while suppression of the nonsense mutation by the aminoglycoside allows the synthesis of a 35-kDa protein.[6] The nonsense mutation context was derived from PCDH15 cDNA. The reporter plasmid was in vitro transcribed and translated in the presence of [^{35}S]methionine, and the reaction products were separated by SDS–PAGE and quantified using PhosphorImager analysis. The suppression level was calculated as the relative proportion of the 35-kDa product out of total protein (the sum of 35-kDa and 25-kDa), and the translation level was calculated as the relative proportion of the total protein at each drug concentration out of the total protein without drug. The results are averages of at least three independent experiments.

(+5), it is possible that **3** has less nonspecific binding to other cell components than those of paromomycin and gentamicin.

Finally, the designed structures **1**–**9** were investigated as antibacterial agents against both Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis*) bacteria (Table 1). From the MIC values, it can be seen that whereas gentamicin and paromomycin exhibit excellent

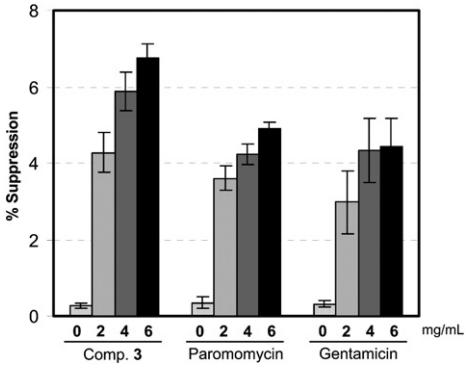


Figure 4. Ex vivo suppression of a nonsense mutation by compound **3**, paromomycin, and gentamicin. The p2Luc plasmid containing a TGA C nonsense mutation in a polylinker between the *renilla* luciferase and firefly luciferase genes[16] was transfected into COS-7 cells and addition of tested compounds was performed after 15 h. Luciferase activity was determined following 24 h of incubation, using the Dual Luciferase Reporter Assay System (Promega). Stop codon read-through was calculated as described previously.[16] The results are averages of at least three independent experiments.

antibacterial activity against both strains, the synthetic derivatives did not retain the antibiotic character of natural aminoglycosides. The antibacterial activities of all new compounds, including compound **3** which had relatively high read-through activity, are markedly lower than that of the parent paromomycin.

The origin of aminoglycoside toxicity is still controversial and probably results from a combination of different factors/mechanisms.[15] Yet, several encouraging results obtained with compound **3** suggest that it might exhibit reduced toxicity relative to that of the parent paromomycin or gentamicin compounds. The data in Table 1 and Figure 3 show that besides the significant read-through activity of compound **3**, it also retained much higher translation level (~72% at 80 µg mL^{-1}) than either gentamicin or paromomycin (~40% at 30–40 µg mL^{-1}). Another factor thought to be one of the causes for the cytotoxicity of aminoglycosides is their binding to mitochondrial 12S rRNA A-site (whose sequence is very close to the bacterial A-site).[8] Thus, the observed inability of **3** to show significant antibacterial activity could also hint about its lower toxicity. Nevertheless, only cell cytotoxicity assay could determine whether these results truly indicate about lower toxicity of compound **3**.

In summary, this study provides a new direction for the development of novel aminoglycoside-based small molecules that selectively target mammalian cells by means of optimizing the efficiency of aminoglycoside-induced suppression of premature stop mutations; this progress may offer promise for the treatment of many genetic diseases. Thus, although the 'ideal readthrough inducer molecule' is yet to be identified, the results introduced in this study indicate that this is an achievable goal. Direct cytotoxicity and cell permeability assays of **3**, along with the design and synthesis of 'second generation derivatives' of paromomycin, are currently underway.

Acknowledgments

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13. Mutations in the *PCDH15* gene (which encodes protocardherin 15) cause type 1 Usher syndrome (USH1), which is characterized by profound prelingual hearing loss, vestibular areflexia, and prepubertal onset of retinitis pigmentosa (RP) (Petit, C. *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 271). In humans, four different *PCDH15* USH1-causing nonsense mutations (R3X, R245X, R643X, and R929X) have been reported. Interestingly, while the above nonsense mutations of *PCDH15* cause USH1, certain missense mutations in the same gene cause only nonsyndromic deafness, which is not associated with RP. Such observations suggest that partial or low level activity of the protein encoded by this gene may be sufficient for normal retinal function, making it a suitable candidate for read-through therapy.
14. Suppression of nonsense mutations by compounds **1**–**9** was tested in vitro using a reporter plasmid harboring the R3X mutation of the *PCDH15* gene (Ahmed, Z. M.; Riazuddin, S.; Bernstein, S. L.; Ahmed, Z.; Khan, S.; Griffith, A. J.; Morell, R. J.; Friedman, T. B.; Riazuddin, S.; Wilcox, E. R. *Am. J. Hum. Genet.* **2001,** *69*, 25). To create this plasmid, the oligonucleotides GATCCATGT TTTGACAGTTTTATCTCTGGACA and AGCTTGTC CAGAGATAAAACTGTCAAAACATG were annealed to each other, and inserted into the *Bam*HI and *Hin*dIII sites of plasmid pDB650.[6]
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