The minor gentamicin complex component, X2, is a potent premature stop codon readthrough molecule with therapeutic potential

Westley J. Friesen*, Briana Johnson, Jairo Sierra, Jin Zhuo, Priya Vazirani, Xiaojiao Xue, Yuki Tomizawa, Ramil Baiazitov, Christie Morrill, Hongyu Ren, Suresh Babu, Young-Choon Moon, Art Branstrom, Anna Mollin, Jean Hedrick, Josephine Sheedy, Gary Elfring, Marla Weetall, Joseph M. Colacino, Ellen M. Welch, Stuart W. Peltz

PTC Therapeutics, South Plainfield, NJ, United States of America

*wfriesen@ptcbio.com

Abstract

Nonsense mutations, resulting in a premature stop codon in the open reading frame of mRNAs are responsible for thousands of inherited diseases. Readthrough of premature stop codons by small molecule drugs has emerged as a promising therapeutic approach to treat disorders resulting from premature termination of translation. The aminoglycoside antibiotics are a class of molecule known to promote readthrough at premature termination codons. Gentamicin consists of a mixture of major and minor aminoglycoside components. Here, we investigated the readthrough activities of the individual components and show that each of the four major gentamicin complex components representing 92–99% of the complex each had similar potency and activity to that of the complex itself. In contrast, a minor component (gentamicin X2) was found to be the most potent and active readthrough component in the gentamicin complex. The known oto- and nephrotoxicity associated with aminoglycosides preclude long-term use as readthrough agents. Thus, we evaluated the components of the gentamicin complex as well as the so-called “designer” aminoglycoside, NB124, for in vitro and in vivo safety. In cells, we observed that gentamicin X2 had a safety/readthrough ratio (cytotoxicity/readthrough potency) superior to that of gentamicin, G418 or NB124. In rodents, we observed that gentamicin X2 showed a safety profile that was superior to G418 overall including reduced nephrotoxicity. These results support further investigation of gentamicin X2 as a therapeutic readthrough agent.

Introduction

The presence of a nonsense mutation in the open reading frame of a gene leads to the introduction of a premature termination codon in the mRNA that results in the production of a truncated-nonfunctional protein product. Nonsense mutations are the cause of approximately 11% of all genetic diseases [1]. Readthrough of premature termination codons to allow
production of full length protein has the potential to treat all patients that harbor nonsense mutations as the cause of their disease. We have identified two novel classes of readthrough compounds. The first, ataluren, selectively induces ribosomal readthrough at premature stop codons across many different disease model systems [2–7]. Ataluren demonstrated activity in clinical trials of nonsense mutation Duchenne muscular dystrophy (DMD) [8–10] and has received conditional marketing approval in the European Union for nonsense mutation DMD [11]. The second compound, clitocine, is a nucleoside analog that induces readthrough by incorporating into RNA, including the site of the premature stop codon and has potential therapeutic utility to treat cancers with nonsense-mutated tumor suppressor genes [12].

It is known that aminoglycoside antibiotics can induce readthrough of premature termination codons via modulation of the ribosome in disease causing genes [13–15]. The most common aminoglycosides analyzed in pre-clinical studies of readthrough are gentamicin and G418. Gentamicin is an antibiotic that is used clinically to treat severe Gram negative bacterial infections despite its potential to induce nephrotoxicity [16] and ototoxicity [17]. In addition to being investigated in many pre-clinical readthrough models, gentamicin has also been investigated in clinical trials of DMD and Cystic Fibrosis (CF) which showed early promising results [18–22]. The aminoglycoside G418 is known to be the most potent and active readthrough compound, and is used extensively in cell-based readthrough assays [23–25]. However, G418 is not used clinically as an antibiotic or as a readthrough agent.

Commercially available gentamicin is composed of a complex of individual aminoglycoside congeners [26–28]. The major components of the gentamicin complex are gentamicins C1, C1a, C2, and C2a constituting 92–99% of the complex. Of the minor components, gentamicin B was found to constitute between 0.8 and 5.3% of the complex, and C2b between 1.3 and 2.1%. Sisomisin was found to be rare, comprising only 0.4–0.6% of the complex. Additionally, gentamicin A and X2 (also known as gentamicin X) together comprised between 1.1 and 7.8% of the mixture. Other components in the complex include gentamicin B1 and garamine.

Here, we investigate the potential to advance an aminoglycoside as a readthrough therapeutic. We report readthrough activity, cytotoxicity and safety of the available gentamicin complex components. We found that all the major components had readthrough activity. One minor component, gentamicin X2, was particularly potent and active. We show in cytotoxicity assays and in tolerability studies in rats that among the aminoglycosides tested, gentamicin X2 may offer the best possibility of achieving a therapeutic window large enough for clinical benefit with limited toxicity.

Results

Gentamicin X2, a minor component of the gentamicin complex, is a potent readthrough compound

Because it is approved for use as an antibiotic, gentamicin has been investigated as a potential readthrough therapeutic in many different nonsense mutation disease models [29]. In a search to identify therapeutic aminoglycosides with reduced toxicity and increased readthrough potency and activity, we evaluated all of the commercially available gentamicin components and compared their biological activity to that of G418 and the so-called “designer” aminoglycosides NB84 and NB124. We utilized HDQ-P1 mammary carcinoma cells, which are homozygous for R213*, a UGA premature termination codon in the gene for the tumor suppressor protein p53 [30]. Aminoglycoside readthrough activity in HDQ-P1 cells resulted in production of full-length p53 protein that was measured using a previously described p53 Meso Scale Discovery (MSD) immunoassay (Fig 1A and 1B) or by western blot analysis (Fig 1C) [12,31]. All of the major gentamicin components (C1, C1a, C2, and C2a) were weakly active with EC2X.
Aminoglycosides as potential readthrough therapeutics

**A**

H D Q - P 1 p 5 U G A 2 1 3 R e a d t h r o u g h

Fold over DMSO

| Concentration (μM) |
|-------------------|
| 1                |
| 10               |
| 100              |
| 1000             |
| 10000            |

**B**

H D Q - P 1 p 5 U G A 2 1 3 R e a d t h r o u g h

Fold over DMSO

| Concentration (μM) |
|-------------------|
| 1                |
| 10               |
| 100              |
| 1000             |
| 10000            |

**C**

| Gentamicin | G418 | Gent X2 | Gent C1 | Gent C2 |
|------------|------|---------|---------|---------|
| PBS        | 10   | 100     | 1000    | 10000   |
| 10         |      |         |         |         |
| 100        |      |         |         |         |
| 1000       |      |         |         |         |
| 1000       |      |         |         |         |

![Western Blot Analysis](image)

- **p53**
- **Actin**

| Gent C1a | Gent C2a | Gent A | Sisomicin | Clitocine |
|----------|----------|--------|-----------|-----------|
| PBS      | 10       | 100    | 1000      | 100       |
| 10       |          |        |           |           |
| 100      |          |        |           |           |
| 1000     |          |        |           |           |
| 1000     |          |        |           |           |

| NB84  | NB127 | NB124 |
|-------|-------|-------|
| PBS   | 10    | 100   |
| 10    |       |       |
| 100   |       |       |
| 1000  |       |       |

- **p53**
- **Actin**
values (the concentration required to double the level of full-length p53 protein) in the triple digit μM range and maximum readthrough activity ranging from 3- to 7-fold above background (Fig 1A and 1C and Table 1). In contrast, we observed that among the three minor components available to us (gentamicin A, X2 and sisomycin) gentamicin X2 was the most active readthrough component inducing full length p53 with an EC$_{2X}$ of 19 ± 6 μM and a maximum fold increase above background of 39 ± 31 (Fig 1B and 1C and Table 1). The readthrough potency of gentamicin X2 was slightly less than that of the most potent and active aminoglycoside known, G418 (EC$_{2X}$ of 9 ± 5, maximum fold induction of 44 ± 19).

Because readthrough potency for a given compound can vary between readthrough assays, we wished to compare the rank order of potency of the aminoglycosides in three different readthrough assays—HDQ-p53$_{UGA13}$ and tGFP-542X cell-based assays and fLuc-190-NS cell-free assay (Table 1). The fLuc-190-NS assay is an in vitro rabbit reticulocyte lysate translation of an fLuc mRNA harboring a UGA nonsense mutation at codon 190 (fLuc-190-NS) [7]. It is believed that positively charged aminoglycosides are poorly taken up by cells, and we used this cell-free readthrough assay to eliminate the potential variable of cell uptake when measuring readthrough potency. Note that all the aminoglycosides tested were more potent in the cell-free readthrough assay (Table 1). As further confirmation of readthrough potency, the previously described tGFP-542X readthrough assay was used [31]. This assay consists of 293H cells stably expressing a construct containing the coding sequence of tGFP followed by codons 539–545 of human CFTR (with codon 542 being UGA) followed by hemagglutinin (HA-tag) and six histidine (HIS-tag) coding sequences. Readthrough protein was detected using a HA-HIS MSD immunoassay described previously [31].

To rank order the compounds, we statistically compared average readthrough potency (EC$_{2X}$) using the 95% confidence limits of the means (Fig 2A–2C) [32;33]. Means were considered statistically different if they fall outside of the 95% confidence limit of the comparator mean and vice versa. NB84 and NB124 were equivalent in potency to gentamicin X2 in all the assays except in fLuc-190-NS where NB84 was less potent. In all three assays, G418 was the most potent. The potency rank order was G418 > gentamicin X2 = NB124 = NB84 > gentamicin, with the exception of NB84 being slightly less potent than NB124 and gentamicin X2 in the fLuc-190-NS cell-free assay.

**Gentamicin X2 has a greater readthrough/safety window than gentamicin, G418 or NB124**

When considering compounds for potential readthrough therapy it is important to monitor cellular and animal safety. In order to determine the most promising compounds to further evaluate in in vivo safety, we measured cytotoxicity in two different cell types. We used activated human peripheral blood mononuclear cells (PBMCs) (Table 2 and Fig 2D and 2E). PBMCs are a primary cell that when activated with phytohaemagglutinin (PHA) expand quickly and are commonly used as an in vitro surrogate for rapidly growing cells in the human body to evaluate cytotoxicity [34]. Prior to advancing compounds into human studies, safety evaluation in rodents is performed. As an in vitro surrogate for rodent toxicity, we used primary mouse myoblasts. We statistically compared average CC$_{50}$ (cytotoxic concentration 50%) using the 95% confidence limits of the means (Fig 2D and 2E) [32;33]. Means were
Table 1. Aminoglycoside potency and activity in readthrough assays.

| Compound   | HDQ-p53UGA213 (EC_{2X}, μM) | HDQ-p53UGA213 (Maxfold) | tGFP-542X (EC_{2X}, μM) | tGFP-542X (Maxfold) | fLuc-190-NS (EC_{2X}, nM) | fLuc-190-NS (Maxfold) |
|------------|-----------------------------|------------------------|------------------------|---------------------|--------------------------|------------------------|
| Genetin    | 9 ± 5                       | 44 ± 19                | 9 ± 4                  | 318 ± 93            | 6.4 ± 0.2                | 53 ± 2                 |
| G418       | 19 ± 6                      | 39 ± 31                | 36 ± 16                | 135 ± 87            | 29 ± 9                   | 195 ± 18               |
| NB84       | 17 ± 5                      | 30 ± 12                | 38 ± 12                | 110 ± 57            | 45 ± 11                  | 162 ± 11               |
| NB124      | 21 ± 6                      | 62 ± 26                | 51 ± 25                | 180 ± 22            | 25 ± 7                   | 180 ± 23               |
| Gentamicin | 418 ± 310                   | 4 ± 1                  | 485 ± 67               | 4 ± 0.8             | 1028 ± 89                | 71 ± 8                 |
| Gentamicin | 311 ± 321                   | 5 ± 3                  | 439 ± 192              | 3 ± 2               | 1061 ± 262               | 19 ± 2                 |
| Gentamicin | 142 ± 86                    | 6 ± 5                  | 266 ± 44               | 9 ± 2               | 1086 ± 763               | 97 ± 12                |
| Gentamicin | 228 ± 159                   | 3 ± 1                  | 715 ± 195              | 2 ± 0.1             | 1123 ± 59                | 19 ± 2                 |
| Gentamicin | 252 ± 49                    | 4 ± 1                  | 583 ± 110              | 3 ± 0.5             | 1077 ± 29                | 32 ± 5                 |
| Gentamicin | 586± 100                    | 3 ± 0.4                | 385 ± 123              | 4 ± 0.4             | 450 ± 15                 | 6.7 ± 0.4              |
| Sisomycin  | >1000                       | 1.3 ± 0.1              | >1000                  | 1 ± 0.1             | 2596 ± 428               | 4 ± 0.5                |

Values are reported as averages plus or minus the standard deviation for at least three separate experiments. [7;12;31]. EC_{2X}: concentration required to double the level of full-length p53 protein. Maxfold: maximum readthrough activity achieved above background. All values used for calculating averages can be found in S1 File. Note: maximum fold values for gentamicin X2 and G418 differ in Table 1 from that in Fig 1B because the highest concentration used for the measurements in Table 1 was 300 μM whereas Fig 1B was 1000 μM.

https://doi.org/10.1371/journal.pone.0206158.t001

considered statistically different if they fall outside of the 95% confidence limit of the comparator mean and vice versa. Statistical comparison of the average CC_{50}s showed that gentamicin X2 was less cytotoxic than G418, NB84 or NB124 in both activated PBMCs and myoblasts (Fig 2D and 2E) indicating that gentamicin X2 is a potentially more promising readthrough compound than the other aminoglycosides.

Aminoglycoside antibiotics exhibit well known toxicities to the kidney and hair cells of the inner ear [16;17]. Toxicity of compounds to zebra fish hair cells (neuromasts) is used as a surrogate for potential human inner ear hair cell cytotoxicity [35–38] We evaluated the effect of the compounds on zebrafish neuromasts, and found no statistical differences between the compounds (Fig 2F, Table 2 and S1 Fig).

Cellular potency of a small molecule drug will determine the concentration needed for therapeutic effect in vivo. When comparing compounds, the ratio of cellular cytotoxicity to readthrough potency (termed safety window) is a surrogate for in vivo therapeutic window (the ratio between efficacious dose and toxic dose). We calculated safety windows by dividing average cellular cytotoxicity values (average CC_{50}s) for all three cytotoxicity assays by readthrough potency (average EC_{2X}s) for all three readthrough assays and determined the 95% confidence limits for each ratio in order to statistically compare compounds. Ratios were considered statistically different if they fall outside of the 95% confidence limit of the comparator ratio and vice versa. Gentamicin X2 had a statistically better safety window (based on 95% confidence limits) than either NB84, NB124 or G418 in PBMCs. The safety window in myoblasts for all compounds (Fig 2J–2L) indicating that gentamicin X2 is a potentially more promising readthrough compound than the other aminoglycosides.

Gentamicin X2 was better tolerated in vivo than was G418

Although G418 has long been known as one of the most active and potent readthrough aminoglycosides due to the combination of its readthrough and mRNA stability functions [24;25;31],
Fig 2. Statistical comparison of selected aminoglycosides. Graphs show average values for at least three independent experiments (open circles) for each aminoglycoside indicated on the y-axis. Error bars indicate the 95% confidence limits for each average. The title of each graph indicates the average measurement or the average calculated ratio. A-C are potency averages from the three readthrough assays; D-F are averages for the three cytotoxicity assays and G-O show the mean ratios between the readthrough and the cytotoxicity assays. All values used for calculating averages can be found in S1 File.

https://doi.org/10.1371/journal.pone.0206158.g002
evaluation of its in vivo safety has lagged behind. We compared the in vivo safety of gentamicin X2 and G418 because they had the most favorable in vitro readthrough and cytotoxicity profiles (Fig 2). We first determined that G418 and gentamicin X2 had similar pharmacokinetic profiles in rats (Fig 3 and Table 3). When administered by subcutaneous injection at a dose of 10 mg/kg, G418 and gentamicin X2 showed similar plasma 24-hour area under the curve (AUC\textsubscript{24}), 15 h μg/mL and 13 h μg/mL, respectively (Fig 3A). Twenty-four hours after dosing, plasma levels of G418 and gentamicin X2 were at or below the lower limit of quantification (~1 ng/g tissue) and low in the quadriceps (~40 ng/g tissue). Levels were much higher in the kidney (Fig 3B), consistent with previous publications demonstrating that aminoglycosides accumulate in the kidney, likely contributing to renal toxicity [39;40]. Kidney levels of G418 were higher than those of gentamicin X2, 12 ± 3 and 7 ± 1 μg/g, respectively.

To evaluate in vivo safety we administered the compounds daily to rats (n = 6 rats per dose group) by subcutaneous injection for 14 days. Toxicokinetics evaluated on Day 1 and Day 14 showed no accumulation of either compound over time (S4 Fig). Gentamicin X2 was well-tolerated at all dose levels compared to G418. (Fig 4). At the highest dose of G418 (20 mg/kg), three rats were either euthanized or found dead and the rats that tolerated the full 14 days of dosing exhibited significant reductions in body weight -35% less than vehicle treated rats by day 14 (Fig 4B). In contrast, gentamicin X2 did not affect body weight at a dose of 20 mg/kg, and only slightly reduced body weight at a dose of 40 mg/kg (Fig 4A). No rats were found dead or euthanized in any of the three X2 dose groups.

We also evaluated hematological parameters and found no change in the numbers of red and white blood cells or platelets in any of the gentamicin X2 dosed groups compared to those rats treated with vehicle alone (S2 Fig).

**Gentamicin X2 caused less kidney damage than G418**

Aminoglycosides are known to cause acute kidney injury in humans (Guthrie 91–96;Wargo and Edwards 573–77). Kidney toxicity is typically measured using creatinine and blood urea nitrogen. On Days 7 and 14, creatinine and blood urea nitrogen (BUN) levels (measures of kidney function) were significantly elevated (p < 0.05, one way ANOVA, multiple comparisons vs vehicle) in rats treated with G418 at a dose of 20 mg/kg (S3 Fig) indicating that G418 was more nephrotoxic than gentamicin X2.

Urine markers for kidney damage were also analyzed in the highest dose groups (gentamicin X2 at 40 mg/kg and G418 at 20 mg/kg) at day 6 and in the mid-level groups (gentamicin X2 at 20 mg/kg and G418 at 10 mg/kg) at day 9. Albumin, clusterin, cystatin C and osteopontin levels were all significantly increased in the groups administered G418. The gentamicin X2 group was less affected, indicating that G418 caused more kidney damage at a dose of 20 mg/kg compared to highest gentamicin X2 group (40 mg/kg) (Fig 5).
Histopathologic analyses were performed on tissues from the kidney, liver and sternum (for bone marrow) at the end of the 14 day dosing period. Histopathology analyses of kidneys from all rats administered G418 at a dose of 20 mg/kg showed tubular necrosis and pelvic dilation. Histopathology analyses of kidneys from rats administered gentamicin X2 at a dose of 40 mg/kg revealed less damage (four of six kidneys had tubular necrosis and pelvic damage) compared to rats administered G418 (Table 4). No histopathology findings were observed in the liver or bone marrow from rats dosed with either gentamicin X2 or G418. Thus, consistent with the urine analysis, rats administered G418 at a dose of 20 mg/kg had more damage to their kidneys than rats administered gentamicin X2 at a dose of 40 mg/kg.

Discussion

Gentamicin is a complex of multiple major and minor components. The readthrough activity, potency, toxicity, and the relative abundance of the individual gentamicin components contribute to overall potency and activity. Greater than 92% of the gentamicin complex is composed of gentamicins C1, C1a, C2, and C2a [26–28]. We found, as expected, that potency of

| Table 3. Pharmacokinetics parameters. |
|--------------------------------------|
|                                      | G418     | Gentamicin X2 |
| T_{max} (hr)                         | 0.33     | 0.5          |
| C_{max} (µg/mL)                      | 7.4      | 5.6          |
| AUC_{Last} (h.µg/mL)                | 15       | 13           |
| Half-life (hr)                       | 1.5      | 2.6          |

https://doi.org/10.1371/journal.pone.0206158.t003
the complex (EC$_{2X}$ = 418 μM ± 310), was similar to that of the major complex components (EC$_{2X}$ range of 142–311 μM) (Table 1). It has been shown recently that gentamicin B1, an unquantified minor component of the complex, has considerably greater readthrough activity and potency than the complex or any of its major components [41]. Although we were unable to obtain gentamicin B1 for testing, we present data showing that another minor component, X2, is also a potent and active readthrough aminoglycoside. The amount of B1 and X2 likely vary between gentamicin preparations and it was therefore proposed that the varying amounts of highly potent complex components could cause variable readthrough activity of different gentamicin preparations [41]. However, because the readthrough activity and potency of the major individual components are similar in magnitude to that of the complex (Table 1), it is also possible that the major components are responsible for the bulk of the readthrough activity of the complex.

As reported previously [24;25], and confirmed here, G418 is the most potent aminoglycoside for readthrough activity. It differs structurally from the majority of gentamicin components by having a hydroxyl group at the C(6') position of ring A (Fig 6). The only structural difference between G418 and X2 is the replacement of the C(7') carbon atom with a hydrogen atom in gentamicin X2. G418 is an isomer of gentamicin B1 in which the OH and NH$_2$ groups at C(2') and C(6') are switched. It is thought that the C(6')-OH group is crucial for readthrough activity in eukaryotic organisms [26;42]. Recent structural work with G418 bound to ribosomal RNA [36] [14;36] indicates that the C(6')-OH group (H-bond acceptor) of G418 and the exocyclic NH$_2$ group (H-bond-donor) of ribosomal guanosine at position 1645 form a hydrogen bond. This hydrogen bond is believed to be crucial for the specific binding of G418 to the eukaryotic ribosome. If the C(6')-OH is replaced by a C(6')-NH$_2$ group, as it is in the major components of the gentamicin complex, the NH$_2$ group will be highly protonated [43] at physiological pH and thus, unable to act as an efficient H-bond acceptor. This likely explains
the lower eukaryotic readthrough potency of the gentamicin complex, which consists predominantly of gentamicins C1, C1a, C2, and C2a, each containing a C(6')-amino group.

Others have prepared and evaluated a series of semisynthetic aminoglycosides and determined that in addition to the nature of the polar group (amine vs hydroxyl) at C(6') the presence and configuration of the C(6')-Me group are important for readthrough activity [44]. The most potent compounds in this series contain a C(6')-methyl group in the (R) configuration at C(6'), in addition to the C(6')-OH group. The same moiety, a secondary alcohol in the R-configuration at C(6'), is present in G418.

Aminoglycosides have been investigated as potential readthrough therapeutics. Gentamicin has been studied both in preclinical and clinical settings [18–22]. Designer aminoglycosides (for example, NB124) have also been synthesized and shown to have readthrough activity with reduced cytotoxicity [45–47]. Recently G418 was shown to be more potent than NB124 in readthrough of five different p53 nonsense codons [23]. This is in agreement with our data (Table 1 and Fig 2) showing that G418 is more potent than NB124. We evaluated neuromast toxicity (CC50) as a surrogate for ototoxicity and found that the CC50 values for gentamicin, NB124, NB84, and gentamicin X2 were 2.8 ± 1.2 μM, 15 ± 4 μM, 8 ± 0.1 μM and 14 ± 7 μM, respectively (Table 2). This compares favorably with CC50 values obtained previously in mouse cochlear explants where gentamicin, NB124, and NB84 had a CC50 values of 3.5 μM, 15 μM and 20 μM, respectively [48]. Although the absolute values differ slightly, the rank order is the same indicating that both assays can be used to rank order the cytotoxicity of compounds.

When considering a compound for readthrough therapy, a sufficient ratio of safety to readthrough potency is critical. Here we show that gentamicin X2 has a greater readthrough-safety window than the other compounds evaluated: NB124, NB84 or G418. Our data demonstrate that gentamicin X2 warrants further investigation to determine its potential clinical utility in treating genetic diseases and cancers caused by nonsense mutations.

**Materials and methods**

**Animal usage**

All in-life animal procedures were performed in a laboratory certified by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) with approval from the Rutgers Institutional Care and Animal Use Committee.

| Table 4. Rat kidney histopathology. |
|-------------------------------------|
|                                     |
| **Vehicle** | X2 (10 mg/kg) | X2 (20 mg/kg) | X2 (40 mg/kg) | G418 (10 mg/kg) | G418 (20 mg/kg) |
| Animals completed study | 6 | 6 | 6 | 6 | 6 | 3 |
| Animals examined | 6 | 6 | 6 | 6 | 6 | 3 |
| **Kidneys** | | | | | | |
| Kidneys examined | 6 | 6 | 6 | 6 | 6 | 3 |
| Mineralization | 0 | 0 | 0 | 0 | 1 | 0 |
| Tubular necrosis | 0 | 2 | 2 | 4 | 4 | 3 |
| Pelvic dilation | 0 | 0 | 1 | 4 | 2 | 3 |

https://doi.org/10.1371/journal.pone.0206158.t004
Aminoglycoside compounds

All compounds used in this study were obtained from TOKU-E (Bellingham, Washington), except for G418, which was obtained from GoldBio Technology (www.goldbio.com).

Protein detection

Western blot hybridization and Meso Scale Discovery (MSD) immunoassay detection of p53 were done as previously described [12]. Detection of tGFP-CFTR<sub>G542X</sub>-HA-HIS protein expressed from the Turbo GFP-CFTR-G542X construct [31] stably incorporated into 293H cells was done using standard MSD immunoassay methods (Rockville, Maryland, https://www.mesoscale.com). Cells (45K per well) were grown in 96-well plates and treated with compounds for 24 hours. Multi-array 96 well plates (MSD, L15XA) were coated with 2 μg/well HA-tag antibody (Invitrogen, 26183) in 30 μl of PBS (phosphate buffer saline, pH 7.6 and 137 mM NaCl) followed by washing three times with PBS-T (PBS with 0.1% tween-20) and blocking for 1 hour with 150 μl of TBS-C (50 mM Tris pH 7.5, 238 mM NaCl, 2.7 mM KCl and 1% Casein). Cells were lysed with 60 μl per well of lysis buffer (20 mM Tris-HCL, pH 7.5, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 0.5% Triton X100) for one hour at room temperature and 30 μl of lysate per well were transferred to the coated plate and incubated overnight at 4 °C. Plates were then washed three times with PBS-T and 30 μl per well of 0.25 μg/ml anti-His-tag antibody (Genescript, A00174) in TBS-C was added for 1 hour at room temperature. Plates were then washed three times with PBS-T and 30 μl of 0.25 μg/ml goat anti-rabbit IgG-Sulfo-tag antibody (MSD) in TBS-C was added and incubated for 1 hour at room temperature. Plates were then washed three times with PBS-T and 150 μl read buffer (MSD) was added. Plates were read immediately in a SECTOR S 600 (MSD).
In vitro fluc-190-NS readthrough assay

This cell-free translation assay used synthetic LUC mRNAs harboring an EMCV IRES and LUC codon 190-UGA premature stop codon prepared using the MegaScript in vitro transcription kit (Ambion). Translation reactions were done with the Rabbit Reticulocyte Lysate System (Promega) with 100 ng RNA and increasing concentrations of aminoglycosides. The amount of luminescence produced was determined after approximately 4 hours using a Viewlux CCD imager (Perkin-Elmer). Fold suppression over background was calculated as (aminoglycoside light units/PBS light units) [7].

Cytotoxicity assays

Frozen human Peripheral Blood Mononuclear Cells (PBMCs) were obtained from ALLCELLS (Alameda, CA, Cat# PB003F) from a single donor. Cells were seeded at 50,000 cells per well in a 96-well plate, in RPMI-1640 medium with 10% fetal bovine serum, PHA (REMED Cat# R308528701 at 20 μg/mL) and IL-2 (BD Pharmingen, Cat#554603 at 40 ng/mL). Plates were incubated at 37°C with 5% CO₂ for 4 hours prior to compound treatment. Compounds were serially diluted and added to the plates. Plates were incubated at 37°C, 5% CO₂ incubator, for 72 hours prior to determinations of ATP levels. Cell viability was determined using a chemiluminescent assay to monitor adenosine triphosphate (ATP) levels in live cells (CellTitre-Glo; CTG; Promega, Cat#G7571). Methods were performed per the manufacturer’s instructions.

Neuromast toxicity assays

All neuromast cytotoxicity assays were done at Biobide–BBD Biophenix SL (Gipuzkoa, Spain). Zebrafish embryos 5 dpf (days post fertilization) were exposed to the drug in 24-well plates, 5 embryos per well, two wells per condition to have a sample size of ten embryos. Compounds were tested at 5 doses. After 24 hours of exposure embryos were incubated in DASPEI (2-(4-(dimethylamino)styryl) -N-Ethylpyridinium Iodide) for neuromast staining. Images of each embryo were taken and neuromasts counted. A compound was classified as ototoxic when the number of neuromasts in the treated group was less than that in the untreated control group (One Way ANOVA, multiple comparisons vs. vehicle). The CC_{50} was defined as the concentration required to reduce the number of neuromasts by 50%.

Pharmacokinetics of aminoglycosides in the rat

Test compounds were dissolved in PBS at 1 mg/mL and administered subcutaneously in a volume of 10 mL/kg to deliver a dose of 10 mg/kg. For each compound, 3 rats were dosed and blood was obtained by retro-orbital bleeding at 0.083, 0.25, 0.5, 1, 2, 4, 7, 24 and hours post-dose. At 24 hours, the rat was euthanized and in addition to blood, quadriceps and kidney tissues were collected for subsequent analysis.

Fourteen day safety study in rats

Male Sprague Dawley rats were purchased from Charles River Laboratories, where 6 weeks old at time of study initiation. Rats were group-housed (3 rats per box) in solid bottom cages. Food and water were provided ad libitum. Rats were dosed daily by subcutaneous injection. Groups included 9 rats dosed with vehicle and 6 rats per dose group for gentamicin X2 or G418. Blood was collected for serum chemistry by retro-orbital bleeds on Day 7 and by terminal cardiac puncture on Day 14. For urine collection, rats were housed in metabolic cages for 6 hours. Toxicokinetics was evaluated on Day 1 and Day 14 (S4 Fig). Blood was obtained by retro-orbital bleeding at 0, 0.25, 0.75, 1.5, 4, 7, 24 hours post-dose. Rats were euthanized on
Day 14, 24 hours after the last dose. Body weights were measured on Days 3, 6, 9, 12 and 14. Endpoints to evaluate kidney function included serum chemistry (BUN and creatinine; Day 7 and Day 14), urine biomarkers (albumin, clusterin, cystatin C, osteopontin, KIM1, and alpha GST; Day 6 and Day 9), hematology (Day 14) and histopathology. For histopathology, kidney, liver, and bone marrow were evaluated. Samples collected for histopathology were sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and examined by a board certified veterinary pathologist. BUN, creatine and hematology parameters were measured using a HESKA veterinary system (Loveland, CO). Urine samples were assayed using MSD electrochemiluminescent immunoassay. Samples were analyzed in triplicate using a Sector Imager 6000 instrument (MSD, Rockville, Maryland). Kits used were the Rat Kidney Injury Panel 1 (albumin, Kim-1, NGAL/lipocalin-2, osteopontin), the Argutus Acute Kidney Injury Panel (α-GST, GSTYb1, RPA-1) and the Clusterin Test Kit (MSD), according to the manufacturer’s instructions. Urinary biomarker concentrations were normalized to urinary creatinine to account for differences in urine production and flow rates [49].

**Compound quantification**

Blood was collected into tubes containing dipotassium ethylenediaminetetraacetic acid (K2 EDTA) as the anticoagulant and rotated at room temperature until the blood was centrifuged and the plasma recovered for subsequent analysis by liquid chromatography with tandem mass spectroscopy (LC-MS/MS). Noncompartmental toxicokinetic parameters were determined using Phoenix WinNonLin version 6.1 (Pharsight Corporation, Carey NC).

The concentrations of test compound in plasma, kidney and muscle were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, the plasma and tissue homogenate samples were treated with acetonitrile-methanol mixture containing an internal standard that is a close analog of the test compounds. The treated plasma and brain homogenate samples were centrifuged and the supernatant was collected and analyzed using electrospray LC-MS/MS.

**Statistical methods**

Readthrough activity and toxicity measurements were analyzed by means of a general linear model. The Tukey-Kramer studentized range test was used to compare all main effect means while maintaining intended alpha levels [32]. Safety windows were calculated by dividing the average toxicity measurements by the average readthrough measurements. Fieller’s theorem was applied to calculate 95% confidence limits on these ratios [33]. Statistical analysis for this paper was generated using SAS/STAT software, Version 9.4 of the SAS System for Windows (SAS Institute Inc.).

**Supporting information**

S1 Checklist. NC3Rs ARRIVE Guidelines Checklist.pdf.
(PDF)

S1 File. All readthrough and cytotoxicity data used in this study.
(XLSX)

S1 Fig. Neuromast cytotoxicity. Zebrafish larvae at 5 days post fertilization were incubated with increasing concentrations of aminoglycosides for 24 hours. The box and whiskers bars show the median ± minimum value to maximum value for 10 replicates.
(EPS)
S2 Fig. Administration of G418 and gentamicin X2 does not overtly deplete red blood cells, hemoglobin, white blood cells, or platelets. Rats were dosed by subcutaneous injection for 14 days with G418 or gentamicin X2 and euthanized approximately 24 hours after the last dose. Complete blood counts were evaluated with no obvious differences across groups. Each symbol represents values from an individual rat. The mean is shown as the line for each group. (EPS)

S3 Fig. Administration of G418 (20 mg/kg) increases BUN and creatinine levels. Symbols represent values for individual rats (6 rats per group). For Day 7, all groups included 6 rats per group. For Day 14, in the group dosed with G418 (20 mg/kg), there were 3 rats surviving. Also included in this group (shown in lighter green, highest values in the group) are data from one rat that was euthanized on Day 12 so that there are 4 symbols shown. For Day 14, all other groups included 6 rats per group. (EPS)

S4 Fig. Toxicokinetics of G418 and gentamicin X2. Compounds levels were determined from retro-orbital bleeds at the indicated time points. Error bars represent standard deviation of the mean from two rats. No accumulation was observed between day 0 and day 13. (EPS)

S1 Table. Rat body weight for 14 day safety study. (EPS)

Author Contributions

Conceptualization: Westley J. Friesen, Ramil Baiazitov, Young-Choon Moon, Marla Weetall, Ellen M. Welch.

Data curation: Westley J. Friesen.

Formal analysis: Westley J. Friesen, Gary Elfring.

Investigation: Westley J. Friesen, Briana Johnson, Jairo Sierra, Jin Zhuo, Priya Vazirani, Xiaojiao Xue, Yuki Tomizawa, Ramil Baiazitov, Christie Morrill, Hongyu Ren, Suresh Babu, Art Branstrom, Anna Mollin, Jean Hedrick, Josephine Sheedy.

Methodology: Westley J. Friesen.

Project administration: Westley J. Friesen, Ramil Baiazitov, Young-Choon Moon, Marla Weetall, Ellen M. Welch.

Supervision: Westley J. Friesen, Ramil Baiazitov, Young-Choon Moon, Marla Weetall, Joseph M. Colacino, Ellen M. Welch, Stuart W. Peltz.

Validation: Westley J. Friesen.

Visualization: Westley J. Friesen.

Writing – original draft: Westley J. Friesen.

Writing – review & editing: Westley J. Friesen.

References

1. Mort M, Ivanov D, Cooper DN, Chuzhanova NA. A meta-analysis of nonsense mutations causing human genetic disease. Hum Mutat 2008 Aug; 29(8):1037–47. https://doi.org/10.1002/humu.20763 PMID: 18454449
2. Du M, Liu X, Welch EM, Hirawat S, Peltz SW, Bedwell DM. PTC124 is an orally bioavailable compound that promotes suppression of the human CFTR-G542X nonsense allele in a CF mouse model. Proc Natl Acad Sci U S A 2008 Feb 12; 105(6):2064–9. https://doi.org/10.1073/pnas.0711795105 PMID: 18272502

3. Gregory-Evans CY, Wang X, Wasan KM, Zhao J, Metcalfe AL, Gregory-Evans K. Postnatal manipulation of Pax6 dosage reverses congenital tissue malformation defects. J Clin Invest 2014 Jan; 124(1):111–6. https://doi.org/10.1172/JCI70462 PMID: 24355924

4. Miller JN, Kovacs AD, Pearce DA. The novel Cln1(R151X) mouse model of infantile neuronal ceroid lipofuscinosis (INCL) for testing nonsense suppression therapy. Hum Mol Genet 2015 Jan 1; 24(1):185–96. https://doi.org/10.1093/hmg/ddu428 PMID: 27329764

5. Peltz SW, Morsy M, Welch EM, Jacobson A. Ataluren as an agent for therapeutic nonsense suppression. Annu Rev Med 2013; 64:407–25. https://doi.org/10.1146/annurev-med-120611-144851 Epub;%202012 Nov 28.:407–25. PMID: 23215857

6. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, et al. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007 May 3; 447(7140):87–91. https://doi.org/10.1038/nature05756 PMID: 17450125

7. Bushby K, Finkel R, Wong B, Barohn R, Campbell C, Comi GP, et al. Ataluren treatment of patients with nonsense mutation dystrophinopathy. Muscle Nerve 2014 Oct; 50(4):477–87. https://doi.org/10.1002/mus.24332 PMID: 25042182

8. Finkel RS, Flanigan KM, Wong B, Bonnemann C, Sampson J, Sweeney HL, et al. Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy. PLoS One 2013 Dec 11; 8(12):e81302. https://doi.org/10.1371/journal.pone.0081302 PMID: 24349032

9. McDonald CM, Campbell C, Torricelli RE, Finkel RS, Flanigan KM, Goemans N, et al. Ataluren in patients with nonsense mutation Duchenne muscular dystrophy (ACT DMD): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 2017 Sep 23; 390(10101):1489–98. https://doi.org/10.1016/S0140-6736(17)31611-2 PMID: 28728956

10. Haas M, Vlcek V, Balabanov P, Salmonson T, Bakchine S, Markey G, et al. European Medicines Agency review of ataluren for the treatment of ambulant patients aged 5 years and older with Duchenne muscular dystrophy resulting from a nonsense mutation in the dystrophin gene. Neuromuscul Disord 2015 Jan; 25(1):5–13. https://doi.org/10.1016/j.nmd.2014.11.011 PMID: 25497400

11. Friesen WJ, Troxta CR, Tomizawa Y, Zhuo J, Johnson B, Sierra J, et al. The nucleoside analog clitocine is a potent and efficacious readthrough agent. RNA 2017 Apr; 23(4):567–77. https://doi.org/10.1261/rna.060236.116 PMID: 28096517

12. Howard M, Frizzell RA, Bedwell DM. Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. Nat Med 1996 Apr; 2(4):467–9. PMID: 8597960

13. Prokhorova I, Altman RB, Djumagulov M, Shrestha JP, Urzhumtsev A, Ferguson A, et al. Aminoglycoside interactions and impacts on the eukaryotic ribosome. Proc Natl Acad Sci U S A 2017 Dec 19; 114(51):E10899–E10908. https://doi.org/10.1073/pnas.1715501114 PMID: 29208708

14. Pranke I, Bidou L, Martin N, Blanchet S, Hatton A, Karri S, et al. Factors influencing readthrough therapy for frequent cystic fibrosis premature termination codons. ERJ Open Res 2018 Jan; 4(1).

15. Guthrie OW. Aminoglycoside-induced nephrotoxicity. J Pharm Pract 2014 Dec; 27(6):573–7. https://doi.org/10.1177/0897190014546836 PMID: 25195523

16. Clancy JP, Bebok Z, Ruiz F, King C, Jones J, Walker L, et al. Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. Am J Respir Crit Care Med 2001 Jun; 163(7):1683–92. https://doi.org/10.1164/ajrccm.163.7.2004001 PMID: 11401894

17. Linde L, Kerem B. Introducing sense into nonsense in treatments of human genetic diseases. Trends Genet 2008 Nov; 24(11):552–63. https://doi.org/10.1016/j.tig.2008.08.010 PMID: 18937996

18. Malik V, Rodino-Klapac LR, Violett L, Mendell JR. Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy. Ther Adv Neurol Disord 2010 Nov; 3(6):379–89. https://doi.org/10.1177/1756285610388693 PMID: 21179598
21. Politano L, Nigro G, Nigro V, Piluso G, Papparella S, Paciello O, et al. Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. Acta Myol 2003 May; 22(1):15–21. PMID: 12966700

22. Wiltschanski M, Miller LL, Shoseyov D, Blau H, Rivlin J, Aviram M, et al. Chronic ataluren (PTC124) treatment of nonsense mutation cystic fibrosis. Eur Respir J 2011 Jul; 38(1):59–69. https://doi.org/10.1183/09031936.00120910 PMID: 21233271

23. Bidou L, Bugaud O, Belakhov V, Baasov T, Namy O. Characterization of new-generation aminoglycoside promoting premature termination codon readthrough in cancer cells. RNA Biol 2017 Mar 4; 14(3):378–88. https://doi.org/10.1080/15476286.2017.1285480 PMID: 28145797

24. Burke JF, Mogg AE. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. Nucleic Acids Res 1985 Sep 11; 13(17):6265–72. PMID: 2995924

25. Wilschanski M, Miller LL, Shoseyov D, Blau H, Rivlin J, Aviram M, et al. Chronic ataluren (PTC124) treatment of nonsense mutation cystic fibrosis. Eur Respir J 2011 Jul; 38(1):59–69. https://doi.org/10.1183/09031936.00120910 PMID: 21233271

26. Deubner R, Schollmayer C, Wienen F. Assignment of the major and minor components of gentamicin for evaluation of batches. Magnetic Resonance in Chemistry 2003 Jul 2; 41(8):589–98.

27. Stypulkowska K, Blazewicz A, Fijalek Z, Sarna K. Determination of Gentamicin Sulphate Composition and Related Substances in Pharmaceutical Preparations by LC with Charged Aerosol Detection. Chromatographia 2010 Dec; 72(11–12):1225–9. https://doi.org/10.1365/s10337-010-1763-y PMID: 21212852

28. Vydrin AF, Shikhaliev IV, Makhortov VL, Shcherenko NN, Kolchanova NV. Component composition of gentamicin sulfate preparations. Pharmaceutical Chemistry Journal (Translation of Khimiko-Farmatsevicheskii Zhurnal) 2003; 37(8):448–50.

29. Keeling KM, Brooks DA, Hopwood JJ, Li P, Thompson JN, Bedwell DM. Gentamicin-mediated suppression of Hurler syndrome stop mutations restores a low level of alpha-L-iduronidase activity and reduces lysosomal glycosaminoglycan accumulation. Hum Mol Genet 2001 Feb 1; 10(3):291–9. PMID: 11159948

30. Wang CS, Goulet F, Lavoie J, Drouin R, Auger F, Champtier S, et al. Establishment and characterization of a new cell line derived from a human primary breast carcinoma. Cancer Genet Cytogenet 2000 Jul 1; 120(1):58–72. PMID: 10913678

31. Roy B, Friesen WJ, Tomizawa Y, Leszyk JD, Zhuo J, Johnson B, et al. Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression. Proc Natl Acad Sci U S A 2016 Nov 1; 113(44):12508–13. https://doi.org/10.1073/pnas.1605336113 PMID: 27702906

32. Tukey J. Exploratory Data Analysis. 1977.

33. Zerbe G. On Fieller’s Theorem and the General Linear Model. The American Statistician 1978; 32(3):103–5.

34. Clemedson C, Ekwall B. Overview of the Final MEIC Results: I. The In Vitro—In Vitro Evaluation. Toxicology in Vitro 1999; 13(4):657–63.

35. Chiu LL, Cunningham LL, Raible DW, Rubel EW, Ou HC. Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol 2008 Jun; 9(2):178–90. https://doi.org/10.1007/s10162-008-0118-y PMID: 18408970

36. Garreau de L, Prokhorova I, Hottcamp W, Rodnina MV, Yusupova G, Yusupov M. Structural basis for the inhibition of the eukaryotic ribosome. Nature 2014 Sep 25; 513(7519):517–22. https://doi.org/10.1038/nature13737 PMID: 25209664

37. Ton C, Parnc C. The use of zebrafish for assessing ototoxic and otoprotective agents. Hear Res 2005 Oct; 208(1–2):79–88. https://doi.org/10.1016/j.heares.2005.05.005 PMID: 16014323

38. Williams JA, Holder N. Cell turnover in neuromasts of zebrafish larvae. Hear Res 2000 May; 143(1–2):171–81. PMID: 10771194

39. Aronoff GR, Potratz ST, Brier ME, Walker NE, Fineberg NS, Giant MD, et al. Aminoglycoside accumulation kinetics in rat renal parenchyma. Antimicrob Agents Chemother 1983 Jan; 23(1):74–8. PMID: 6830211

40. Nagai J, Takano M. Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab Pharmacokin 2004 Jun; 19(3):159–70. PMID: 15499183

41. Baradaran-Heravi A, Niesser J, Balgi AD, Choi K, Zimmerman C, South AP, et al. Gentamicin B1 is a minor gentamicin component with major nonsense mutation suppression activity. Proc Natl Acad Sci U S A 2017 Mar 28; 114(13):3479–84. https://doi.org/10.1073/pnas.1620982114 PMID: 28289221
42. Shalev M, Baasov T. When Proteins Start to Make Sense: Fine-tuning Aminoglycosides for PTC Suppression Therapy. Medchemcomm 2014 Aug 1; 5(8):1092–105. https://doi.org/10.1039/C4MD00081A PMID: 25147726

43. Szilagyi L, Pusztahelyi ZS, Jakab S, Kovacs I. Microscopic protonation constants in tobramycin. An NMR and pH study with the aid of partially N-acetylated derivatives. Carbohydr Res 1993 Sep 2; 247:99–109. PMID: 8221735

44. Kandasamy J, Atia-Glikin D, Shulman E, Shavit M, Belakhov V, et al. Increased selectivity toward cytoplasmic versus mitochondrial ribosome confers improved efficiency of synthetic aminoglycosides in fixing damaged genes: a strategy for treatment of genetic diseases caused by nonsense mutations. J Med Chem 2012 Dec 13; 55(23):10630–43. https://doi.org/10.1021/jm3012992 PMID: 23148581

45. Hainrichson M, Nudelman I, Baasov T. Designer aminoglycosides: the race to develop improved antibiotics and compounds for the treatment of human genetic diseases. Org Biomol Chem 2008 Jan 21; 6 (2):227–39. https://doi.org/10.1039/b712690p PMID: 18174989

46. Shalev M, Baasov T. When Proteins Start to Make Sense: Fine-tuning Aminoglycosides for PTC Suppression Therapy. Medchemcomm 2014 Aug 1; 5(8):1092–105. https://doi.org/10.1039/C4MD00081A PMID: 25147726

47. Shulman E, Belakhov V, Wei G, Kendall A, Meyron-Holtz EG, Ben-Shachar D, et al. Designer aminoglycosides that selectively inhibit cytoplasmic rather than mitochondrial ribosomes show decreased ototoxicity: a strategy for the treatment of genetic diseases. J Biol Chem 2014 Jan 24; 289(4):2318–30. https://doi.org/10.1074/jbc.M113.535898 PMID: 24302717

48. Xue X, Mutyam V, Tang L, Biswas S, Du M, Jackson LA, et al. Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor. Am J Respir Cell Mol Biol 2014 Apr; 50(4):805–16. https://doi.org/10.1165/rcmb.2013-0282OC PMID: 24251786

49. Ralib AM, Pickering JW, Shaw GM, Devarajan P, Edelstein CL, Bonventre JV, et al. Test characteristics of urinary biomarkers depend on quantitation method in acute kidney injury. J Am Soc Nephrol 2012 Feb; 23(2):322–33. https://doi.org/10.1681/ASN.2011040325 PMID: 22095948