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## Clinical doses of amikacin provide more effective suppression of the human *CFTR*-G542X stop mutation than gentamicin in a transgenic CF mouse model

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**Abstract** Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause the disease cystic fibrosis. We previously reported that gentamicin administration suppressed a *CFTR* premature stop mutation in a *Cftr*<sup>-/-</sup> mouse model carrying a human *CFTR*-G542X (*hCFTR*-G542X) transgene, resulting in the appearance of hCFTR protein and function. However, the high doses used in that study resulted in peak serum levels well beyond the levels typically administered to humans. To address this problem, we identified doses of both gentamicin and amikacin that resulted in peak serum levels within their accepted clinical ranges. We then asked whether these doses could suppress the *hCFTR*-G542X mutation in the *Cftr*<sup>-/-</sup> *hCFTR*-G542X mouse model. Our results indicate that low doses of each compound restored some hCFTR protein expression and function, as shown by immunofluorescence and short-circuit current measurements. However, we found that amikacin suppressed the *hCFTR*-G542X premature stop mutation more effectively than gentamicin when administered at these clinically relevant doses. Because amikacin is also less toxic than gentamicin, it may represent a superior choice for suppression therapy in patients that carry a premature stop mutation in the *CFTR* gene.



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### Introduction

A large number of diseases including cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), beta thalassemia, and many types of cancers are caused by premature stop mutations. According to the Human Gene Mutation Database, 12% of all mutations reported are single-point mutations that result in premature stop codons [1]. Furthermore, the phenotypes associated with recessive diseases caused by premature stop mutations are frequently more severe than those that result from missense mutations, as premature stop mutations often result in a complete loss of the effected protein and its function. One way to treat

diseases caused by in-frame stop mutations is to reduce the efficiency of translation termination at premature stop codons so that the expression of some full-length, functional protein is restored. Suppression of a premature stop codon occurs when the stop mutation located in the ribosomal A site base pairs with a near-cognate aminoacyl-tRNA (complementary to two of the three nucleotides of a stop codon), which allows an amino acid to be incorporated into the growing polypeptide chain [2, 3]. This leads to continued translation elongation in the correct reading frame and production of the full-length polypeptide.

Aminoglycosides are effective antibiotics due to their ability to tightly bind to bacterial ribosomes and inhibit protein synthesis. However, a number of studies have shown that a subset of aminoglycosides also have the ability to bind weakly to mammalian ribosomes and suppress premature stop mutations [4, 5]. A number of investigations have shown that some aminoglycosides can suppress disease-causing premature stop mutations in mammalian cells and partially restore the expression of functional proteins [5–19]. For example, gentamicin has been shown to partially restore the expression of functional proteins in mouse models of DMD [10] and CF [20] that carry nonsense mutations in the *dystrophin* and cystic fibrosis transmembrane conductance regulator (*CFTR*) genes, respectively. Several small clinical trials have also provided preliminary evidence that aminoglycosides can suppress premature stop mutations in patients with DMD [21] and CF [22–24]. While these preliminary results are promising, it remains to be determined whether the level of *CFTR* protein restored is sufficient to provide a therapeutic benefit to patients with these diseases.

Studies with mouse models are frequently used to test both the safety and efficacy of a potential therapeutic approach before much more expensive clinical trials are undertaken. We previously reported that the aminoglycoside gentamicin is capable of suppressing *CFTR* premature stop mutations in a CF transgenic mouse model, in which an *hCFTR* cDNA containing the G542X premature stop mutation was expressed under the control of the rat intestinal fatty acid binding protein (*FABP*) promoter in a *Cftr*<sup>-/-</sup> mouse. [20]. In that study, the intestine-specific *FABP* promoter was used, as the most severe phenotype associated with CF mice is intestinal blockage that leads to high mortality upon weaning [25]. The lack of lung expression using the *FABP* promoter was not considered to be important for that study, as lung disease is not observed in most CF mouse models, presumably due to the expression of alternate chloride channels in the lung epithelia. This *hCFTR*-G542X *Cftr*<sup>-/-</sup> mouse was used to show that once daily administration of 34 mg/kg of gentamicin or tobramycin via subcutaneous injections could suppress the G542X nonsense mutation and partially restore expression of functional h*CFTR* protein [20]. In that study, gentamicin was found to induce a significantly higher level of functional h*CFTR* protein than tobramycin.

An important concern for the clinical use of aminoglycosides is the side effects associated with their use, which include kidney damage and hearing loss. Because of their

potential toxicity, administration protocols have been developed that maintain aminoglycoside serum concentrations within a clinically acceptable range of recommended peak and trough levels. In a previous study, we showed that gentamicin suppressed the *hCFTR*-G542X nonsense mutation in vivo, although peak serum concentrations used were well above the levels allowed during the recommended clinical use of these compounds [20]. Thus, it was not clear whether an effective level of suppression could be accomplished at concentrations within a safe clinical range.

In the current study, we examined whether gentamicin and amikacin could suppress the G542X mutation when administered at doses within the recommended therapeutic range of serum peak and trough levels. We obtained evidence that both compounds could suppress the *hCFTR*-G542X mutation in the *Cftr*<sup>-/-</sup> mouse model and partially restore functional *CFTR* expression when administered at doses that produced serum levels in the recommended range. However, our results indicate that amikacin suppressed the *hCFTR*-G542X mutation in vivo much more effectively than gentamicin under these conditions, suggesting that it may represent a better choice for the suppression of the *hCFTR*-G542X stop mutation (and possibly other premature stop mutations that cause CF).

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## Materials and methods

### Plasmid construction and generation of transgenic mice

Expression of the *hCFTR* cDNA transgene that contained the G542X (UGA) premature stop mutation was driven by the rat *FABP* promoter. The plasmid construction of the *FABP*-*hCFTR*-G542X transgene and generation of the *Cftr*<sup>-/-</sup> *hCFTR*-G542X were described previously [20].

### In vitro translation system

A modified form of a readthrough reporter system, previously used to monitor the suppression of stop mutations by aminoglycosides [4, 5], was used to determine whether amikacin (Calbiochem) could suppress the *hCFTR*-G542X mutation in an in vitro rabbit reticulocyte lysate (RRL) translation system (Promega TNT system). RNA synthesized from these constructs was driven from the *SP6* promoter. The transcribed reporter gene consists of three major components: an initial open reading frame encoding a 25-kDa protein, a readthrough cassette that contains the stop mutation and surrounding sequence context to be tested for suppression, and an open reading frame downstream of the readthrough cassette that encodes another 10-kDa of protein. In the present study, the readthrough cassette contained the *hCFTR*-G542X premature stop codon and six upstream and downstream codons from the *hCFTR* gene. A DNA fragment containing these 13 codons was introduced into the *Bam*HI and *Hind*III sites of the readthrough cassette region of pDB654

by ligation of the annealed oligonucleotides DB809 and DB810. The resulting plasmid, pDB664, was then transcribed and translated in the RRL translation system in the presence or absence of aminoglycosides. Translation products were monitored by the incorporation of  $^{35}\text{S}$ -methionine into protein followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Efficient translation termination at the G542X stop mutation produced a 25-kDa protein, while suppression of the stop codon resulted in the synthesis of a 35-kDa protein. PhosphorImager analysis (GE Healthcare) was used to determine the relative abundance of each translation product. Suppression of the G542X mutation was expressed as percent readthrough, which was calculated as the  $[35\text{ kda protein}/(25\text{ kda} + 35\text{ kda proteins})] \times 100$ .

#### Aminoglycoside treatment

Aminoglycoside treatment of homozygous *Cftr*<sup>-/-</sup> mice carrying the *FABP-hCFTR-G542X* transgene was initiated 16 days after birth (1 week before weaning). Whenever possible, littermates were divided into aminoglycoside treatment and control groups. The treatment group was given a once daily subcutaneous injection of gentamicin at 34 mg/kg body weight or 5 mg/kg body weight or a once daily subcutaneous injection of amikacin at 170 mg/kg body weight or 15 mg/kg body weight. The mouse pups were weaned at 23 days after birth. Because of the potential for intestinal blockage, mice were maintained on a liquid diet (Peptamen Complete Elemental Diet, Nestle) after weaning. Treatment was continued for 2–3 weeks before killing the animal and harvesting tissues for analysis.

To determine serum levels of gentamicin and amikacin, orbital blood was obtained from age-matched mice at various times after subcutaneous injection of the aminoglycoside and assayed by fluorescence polarization immunoassay (FPIA).

#### Immunofluorescence

Immunofluorescence experiments were carried out essentially as previously described [20]. Immediately after killing the animal, the intestinal tissue was placed in a cryomold (Miles Laboratories, Naperville, IL) containing optimum cutting temperature embedding medium. Samples were flash-frozen by immersion in a metal cup filled with 2-methylbutane that was prechilled with liquid nitrogen. Frozen blocks were then sectioned using a cryostat. Five-micrometer sections were collected and fixed in 3% formaldehyde in phosphate-buffered saline (PBS) for 45 min at room temperature. After blocking with 25–50% normal goat serum for 20 min at room temperature to reduce nonspecific antibody binding, samples were incubated with a 1/200 dilution of the hCFTR polyclonal rabbit antiserum #4562. The antigen used to raise this antiserum included hCFTR NBD1 and a portion of the R domain (amino acids 521–828) fused to the *Escherichia*

*coli* TrpE protein [26]. After incubation with this antibody for 1 h at 37°C, the samples were washed with four changes of PBS and again blocked with 25–50% normal goat serum for 20 min at room temperature. The samples were then incubated for 1 h at 37°C with 25 µg/ml goat anti-rabbit IgG conjugated to AlexaFluor-488 (Molecular Probes #A-11001). Finally, samples were washed twice with PBS and incubated with 20 µg/ml Hoechst 33258 for 4 min at room temperature. To confirm the specificity of the signal obtained, samples were incubated with preimmune rabbit serum in place of the hCFTR-specific serum using the same protocol.

#### Short-circuit current measurement

Four intestinal tissue segments, each approximately 5 mm in length (one from the duodenum, two from the jejunum, and one from the ileum), were dissected and placed in the PBS solution containing Tetrodotoxin ( $3.3 \times 10^{-4}$  µM) for at least 10 min to block sodium channels activated by action potentials. The intestinal segments were mounted as a flat sheet in a modified Ussing chamber (area of ~0.16 cm<sup>2</sup>) and short-circuit recordings were made as previously described [27]. The mucosal bathing solution (37°C, pH 7.4) contained 167.2 mM Na<sup>+</sup>, 5 mM K<sup>+</sup>, 6 mM Cl<sup>-</sup>, 1.2 mM Ca<sup>2+</sup>, 1.2 mM Mg<sup>2+</sup>, 25 mM HCO<sub>3</sub><sup>-</sup>, 4.2 mM PO<sub>4</sub>, and 10.8 mM D-glucose. The serosal surface of the tissue was bathed in a Ringer's solution (37°C, pH 7.4) containing 145 mM Na<sup>+</sup>, 5 mM K<sup>+</sup>, 124.8 mM Cl<sup>-</sup>, 1.2 mM Ca<sup>2+</sup>, 1.2 mM Mg<sup>2+</sup>, 25 mM HCO<sub>3</sub><sup>-</sup>, 4.2 mM PO<sub>4</sub>, and 10 mM D-glucose. Both the mucosal and serosal solutions were constantly circulated by bubbling 95% O<sub>2</sub>/5% CO<sub>2</sub> through the solutions. Forskolin (10 µM) was added to both the mucosal and serosal solutions for at least 10 min, while the short-circuit current was continuously monitored. In all experiments, the current measurements obtained immediately before and 10 min after forskolin addition were used to calculate the current change in each sample.

## Results

Amikacin suppresses the hCFTR-G542X premature stop mutation in a mammalian in vitro translation system

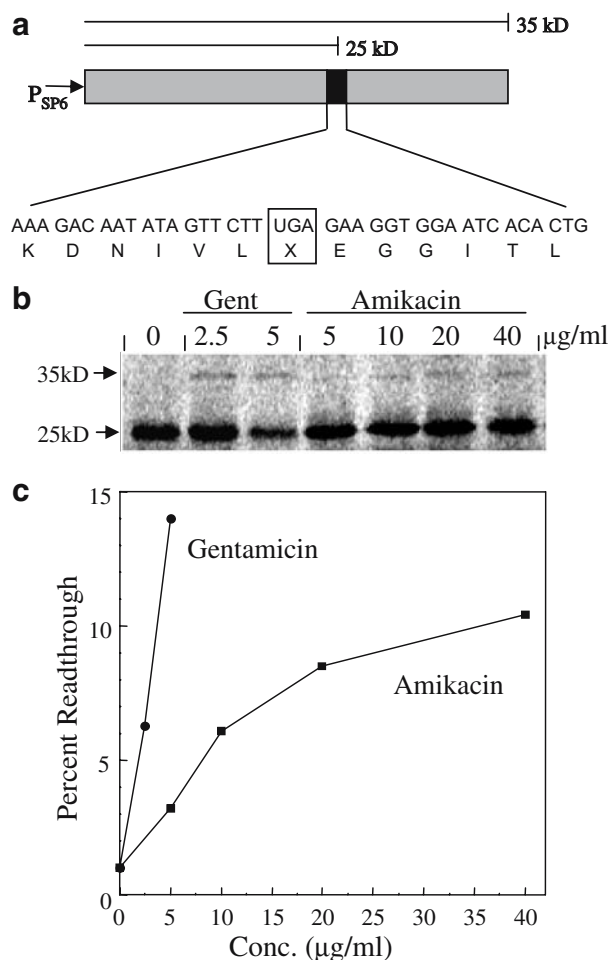
The ability of an aminoglycoside to suppress a premature stop mutation depends on several factors, including the specific features of its chemical structure, the identity of the stop codon to be suppressed, and the sequence context surrounding the stop codon in the mRNA [4, 5]. Given these considerations, the efficiency with which a particular stop mutation is suppressed by aminoglycosides requires some measure of empirical analysis for optimal results. Although amikacin has been shown to suppress premature stop mutations in several mammalian mRNAs in vitro [5, 17], it has not yet been shown to suppress the CFTR-G542X mutation in vitro. We previously reported that

gentamicin and tobramycin suppressed the *hCFTR*-G542X mutation in a mammalian RRL translation system [20]. The in vitro RRL system is useful to obtain an estimate of the effective intracellular concentration required to induce suppression, as well as the concentration above which total protein synthesis is inhibited. Accordingly, we first asked whether amikacin, an aminoglycoside commonly used to treat bacterial infections, could also suppress the *hCFTR*-G542X mutation using the RRL translation system.

Initially, we cloned the *hCFTR*-G542X premature stop codon and six flanking codons on either side into a readthrough reporter plasmid (Fig. 1) [20]. In this reporter system, a 25-kDa protein is produced if termination occurs at the G542X premature stop mutation, while a 35-kDa protein is produced if the G542X stop codon is suppressed. This construct was expressed in a RRL-based coupled transcription/translation system in the presence of increasing concentrations of gentamicin or amikacin, and the concentration of each aminoglycoside that induced the maximum level of readthrough of the G542X mutation (without inhibiting total protein synthesis) was determined. While both compounds were found to suppress termination at the G542X mutation, gentamicin induced readthrough at much lower concentrations than amikacin. The maximum level of G542X readthrough induced by amikacin (11%) was similar to that induced by gentamicin (14%). However, a much higher level of amikacin (40  $\mu\text{g/ml}$ ) than gentamicin (5  $\mu\text{g/ml}$ ) was required to reach these maximum levels of readthrough. These findings suggest that gentamicin may bind to the mammalian ribosome with a higher affinity than amikacin. By the same token, a much lower level of gentamicin also inhibited total protein synthesis in the in vitro translation system (as indicated by the decrease in abundance of the 25-kDa protein band in the reaction that contained 5  $\mu\text{g/ml}$  gentamicin). In contrast, amikacin did not inhibit protein synthesis when it was included in reactions at concentrations as high as 40  $\mu\text{g/ml}$ . These results demonstrate that amikacin can effectively suppress the *hCFTR*-G542X mutation without inhibiting total protein synthesis as observed with gentamicin.

Determining doses of gentamicin and amikacin that produce peak serum levels within the accepted clinical range

The most common forms of aminoglycoside toxicity are kidney damage and hearing loss. Because of the toxic side effects associated with aminoglycoside use, the range of serum levels of these compounds that occur after their administration is carefully monitored. According to the *Physicians Desk Reference* [28], a maximum peak serum concentration of 12  $\mu\text{g/ml}$  (12 mcg/ml) gentamicin is allowed when administered by the intravenous route three times a day. Because amikacin is less toxic than gentamicin, a maximum peak serum concentration of 35  $\mu\text{g/ml}$  amikacin is recommended when using the same administration protocol. More recently, the administration of aminoglycosides once daily with the same total daily



**Fig. 1** Gentamicin and amikacin induce readthrough in an in vitro translation system. **a** Schematic of readthrough reporter system. The *hCFTR*-G542X UGA mutation (boxed) and surrounding context are indicated by the expanded sequence. Termination at the G542X mutation results in a 25-kDa protein, while suppression of that mutation allows continued elongation and the production of a 35-kDa protein. **b** Data showing suppression of the G542X mutation in an RRL in vitro translation system. **c** Quantitation of G542X readthrough from data shown in **b**

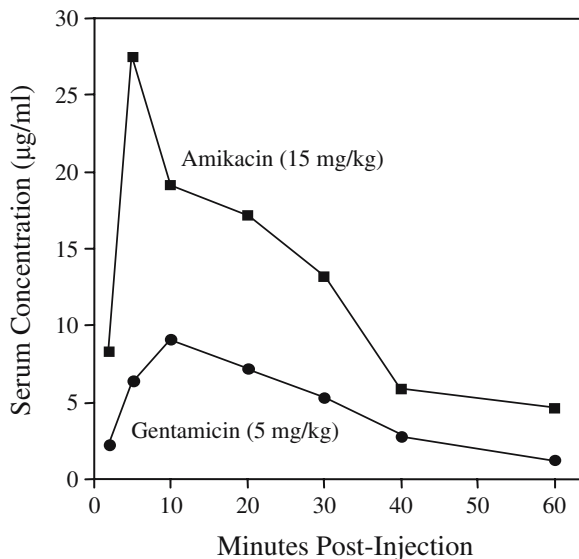
dose previously given in three injections has become increasingly common, as this regimen provides effective antibacterial activity while reducing the incidence of toxicity [29].

In a previous study, we reported that the subcutaneous injection of 34 mg/kg gentamicin once daily suppressed the G542X mutation in *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice and restored a significant level of functional hCFTR protein [20]. However, the peak serum gentamicin concentration measured in the treated mice (64  $\mu\text{g/ml}$ ) was well beyond the peak serum levels recommended for human use. In the current study, we sought to determine whether aminoglycosides could suppress the *hCFTR*-G542X mutation in our mouse model when administered at concentrations that produced serum levels well below the maximum recommended for humans. To determine an acceptable dose of each aminoglycoside, mice were injected subcutaneously with various concentrations of each compound. Blood

samples were then collected at specified times after administration and serum concentrations were determined using FPIA (Fig. 2). We found that the administration of 5 mg/kg gentamicin resulted in a peak serum concentration of 9  $\mu\text{g/ml}$  gentamicin roughly 10 min after the injection. The serum level dropped to only 3  $\mu\text{g/ml}$  by 40 min after injection. In mice administered with 15 mg/kg of amikacin by subcutaneous injection, a peak serum concentration of 27  $\mu\text{g/ml}$  amikacin was reached within 10 min of administration. Again, the level dropped rapidly to a concentration of 6  $\mu\text{g/ml}$  by 40 min after the injection. Similar peak serum levels were obtained when either C57BL/6J or BALB/c mice were administered these doses of gentamicin and amikacin, confirming the general nature of these results in different mouse strains (data not shown). Because these doses yielded peak serum concentrations below the maximum levels recommended for administration three times daily [28], they should have a wide margin of safety for once daily administration. As such, they were used in the experiments described below.

#### A clinical dose of gentamicin suppresses the *hCFTR*-G542X mutation but restores only a barely detectable amount of hCFTR protein

We initially used an immunofluorescence assay to ask whether a once daily, low dose of 5 mg/kg gentamicin can suppress the *hCFTR*-G542X mutation. We previously found that hCFTR protein expression in this transgenic mouse was most prominent at the apical surface of epithelial cells of the submucosal glands in the duodenum after treatment with 34 mg/kg gentamicin [20]. A similar immunofluorescence assay was performed using intestinal



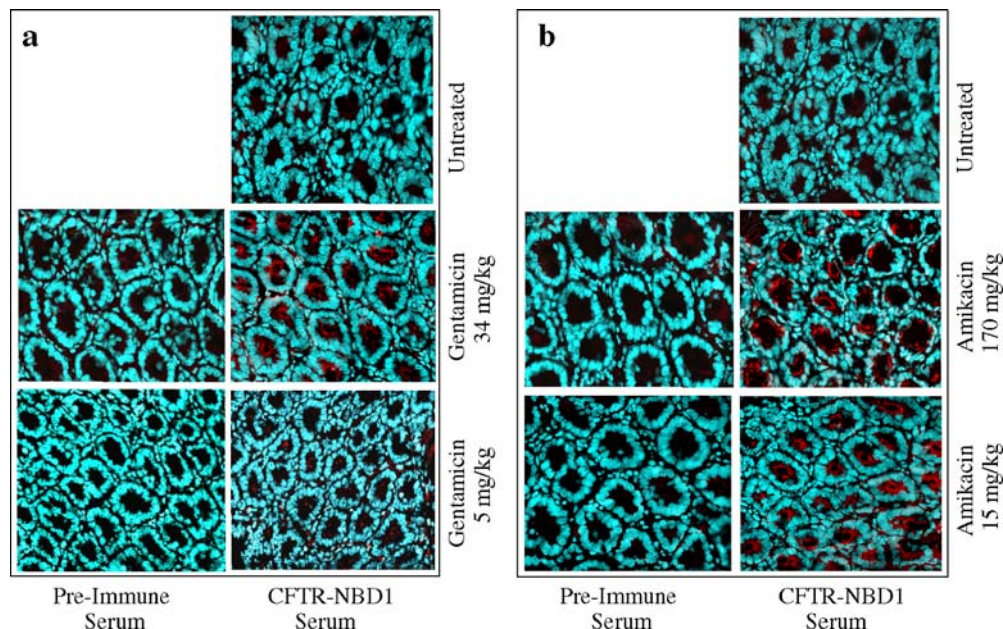
**Fig. 2** Serum levels of gentamicin and amikacin measured after subcutaneous injection. A single blood sample was taken from different age-matched mice at the indicated times after injection of 5 mg/kg of gentamicin or 15 mg/kg of amikacin. The aminoglycoside concentration in each serum sample was determined by fluorescence polarization immunoassay

tissues from untreated *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice or from mice treated with either 34 mg/kg or 5 mg/kg gentamicin (Fig. 3a). No hCFTR protein was detected in samples treated with preimmune serum, demonstrating the specificity of the hCFTR antibody. In the samples treated with the hCFTR-specific antiserum, no hCFTR protein was detected in the intestinal tissue from untreated *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice, while a strong hCFTR protein signal was detected at the apical surface of epithelial cells in the submucosal glands of the duodenum from mice treated with 34 mg/kg gentamicin. We observed a much weaker signal at the same location in submucosal glands of the duodenum from mice treated with 5 mg/kg gentamicin. These results suggest that this lower, clinically relevant dose of gentamicin can suppress the G542X mutation and restore hCFTR protein in *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice, although the level of hCFTR protein detected was substantially less than was observed in animals treated with the higher dose.

#### A clinical dose of amikacin suppresses the *hCFTR*-G542X mutation and restores a significant amount of hCFTR protein expression

We next asked whether amikacin could suppress the *hCFTR*-G542X mutation in the *Cftr*<sup>-/-</sup> *hCFTR*-G542X mouse. To our knowledge, amikacin has not previously been tested for the ability to suppress stop mutations in any in vivo study. Because 34 mg/kg gentamicin was well-tolerated by *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice in our previous study [20] and the doses of amikacin routinely recommended for human use are several folds higher than gentamicin [28], we initially chose to compare a high dose of 170 mg/kg amikacin and a low dose of 15 mg/kg amikacin. We found that once daily subcutaneous injections of mice for 2 to 3 weeks with the high dose of amikacin did not induce any observable adverse effects in treated mice. To determine the ability of amikacin to suppress the *hCFTR*-G542X mutation, the same administration protocol was used and intestinal tissues from mice were assayed by immunofluorescence using either preimmune serum or an hCFTR-specific polyclonal antiserum. No hCFTR protein was detected in samples from untreated *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice using hCFTR-specific antiserum or in samples from treated mice using preimmune serum. However, strong hCFTR protein staining was detected at the apical surface of epithelial cells in the submucosal glands of the duodenum from mice treated with 170 mg/kg amikacin (Fig. 3b). Notably, strong hCFTR protein staining was also detected at the same location in the submucosal glands of the duodenum from mice treated with the much lower dose of 15 mg/kg amikacin. These results indicate that amikacin can restore a significant amount of hCFTR protein in the *Cftr*<sup>-/-</sup> *hCFTR*-G542X mice when administered at either a very high dose or at a lower dose that produces a peak serum level well within the recommended clinical range.

**Fig. 3** hCFTR immunofluorescence in submucosal glands of intestinal tissues from mice treated with gentamicin or amikacin. **a** Samples from the duodenum of homozygous *Cftr*<sup>-/-</sup>*hCFTR-G542X* mice (untreated or treated with 5 or 34 mg/kg gentamicin). **b** Samples from the duodenum of homozygous *Cftr*<sup>-/-</sup>*hCFTR-G542X* mice (untreated or treated with 15 or 170 mg/kg amikacin). Samples were incubated with either preimmune or hCFTR-NBD1 serum. After incubation of the sample with a fluorescent secondary antibody, the samples were visualized by fluorescence microscopy. Other details are described in the “Materials and methods”



Analysis of hCFTR activity in *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice after treatment with clinical doses of gentamicin or amikacin

The hCFTR protein is a cyclic adenosine monophosphate (cAMP)-activated chloride channel that facilitates transepithelial chloride conductance upon activation by cAMP agonists such as forskolin. We previously demonstrated that cAMP-dependent transepithelial chloride conductance could be detected in intestinal tissues of *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice that had been treated with 34 mg/kg gentamicin [20]. We next asked whether lower doses of gentamicin or amikacin that produce peak serum levels within the recommended clinical range could restore cAMP-dependent transepithelial chloride currents. Figure 4 shows representative short-circuit current tracings obtained from *Cftr*<sup>-/-</sup> *hCFTR-G542X* mouse intestinal tissues that were harvested from untreated *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice, or from *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice treated once daily with either 5 mg/kg gentamicin or 15 mg/kg amikacin

for 2 to 3 weeks. It can be seen that forskolin stimulated a significant increase in short-circuit currents in treated animals as compared to untreated controls.

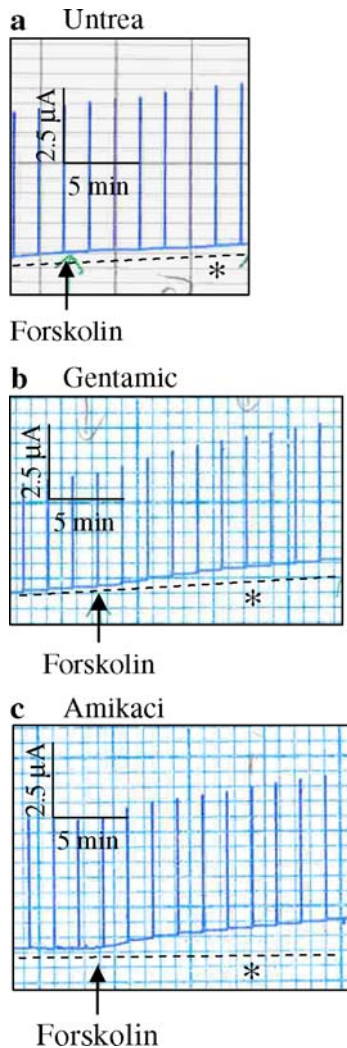
Table 1 provides a summary of data collected from short-circuit current tracings of intestinal tissues harvested from untreated *Cftr*<sup>+/+</sup> *hCFTR-G542X* mice, *+/+* *hCFTR-G542X* mice treated with low doses of gentamicin or amikacin, untreated *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice, and *Cftr*<sup>-/-</sup> *hCFTR-G542X* mice treated with high or low doses of gentamicin or amikacin. We observed cAMP-stimulated short-circuit currents in 100% of the samples (12/12) from untreated *Cftr*<sup>+/+</sup> mice, which were used as positive controls. Short-circuit current measurements from these tissues resulted in an average current of 5.91  $\mu\text{A}/\text{cm}^2$ . We also detected cAMP-stimulated short-circuit currents in 100% of samples from *Cftr*<sup>+/+</sup> mice treated with 5 mg/kg gentamicin (average current 6.24  $\mu\text{A}/\text{cm}^2$ ) or 15 mg/kg amikacin (average current 5.93  $\mu\text{A}/\text{cm}^2$ ). These values obtained with these treated *Cftr*<sup>+/+</sup> mice were not significantly different from untreated *Cftr*<sup>+/+</sup> mice.

**Table 1** Quantitation of cAMP-activated chloride currents in intestinal epithelia

	<i>Cftr</i> <sup>+/+</sup>			<i>Cftr</i> <sup>-/-</sup>				
	Untreated	Gentamicin (5 mg/kg)	Amikacin (15 mg/kg)	Untreated	Gentamicin (34 mg/kg)	Gentamicin (5 mg/kg)	Amikacin (170 mg/kg)	Amikacin (15 mg/kg)
Positive/total signals	12/12	12/12	10/10	1/12	5/8	7/20	9/12	6/15
Percent positive signals	100%	100%	100%	8%	63%	35%	75%	40%
Average current <sup>a</sup>	5.91	6.24	5.93	0.2	1.67	0.82	1.77	1.31
<i>P</i> value <sup>b</sup>	–	0.43	0.49	–	0.023	0.046	0.003	0.027

<sup>a</sup>In all experiments, changes in short-circuit current were measured 10 min after forskolin addition

<sup>b</sup>*P* values comparing the treated with the untreated samples were calculated using the Student's *t* test. A *P*<0.05 value was considered as significant



**Fig. 4** Representative short-circuit current tracings from mouse intestinal tissues. **a** Tracing from the ileum of a homozygous *Cfr*<sup>-/-</sup> *hCFTR*-G542X mouse without any prior aminoglycoside treatment. **b** Tracing from the ileum of a homozygous *Cfr*<sup>-/-</sup> *hCFTR*-G542X mouse after 5 mg/kg gentamicin treatment once daily for 2–3 weeks. **c** Tracing from the ileum of a homozygous *Cfr*<sup>-/-</sup> *hCFTR*-G542X mouse after 15 mg/kg amikacin treatment once daily for 2–3 weeks. The area of the Ussing chamber was 0.16 cm<sup>2</sup>. Scale depicts the current/time relationship. After a baseline current was established, forskolin was added to increase intracellular cAMP levels. Changes in short-circuit current were measured 10 min later (indicated by an asterisk) to ensure that any change in the current was sustained

In untreated *Cfr*<sup>-/-</sup> *hCFTR*-G542X mice used as negative controls, we observed a cAMP-stimulated short-circuit current in only 8% of the samples (1/12), resulting in an average current of only 0.2  $\mu\text{A}/\text{cm}^2$ . In our experimental samples, we found that 63% of samples (5/8) from *Cfr*<sup>-/-</sup> *hCFTR*-G542X mice treated with 34 mg/kg gentamicin yielded cAMP-activated short-circuit currents after forskolin addition, resulting in an average current of 1.67  $\mu\text{A}/\text{cm}^2$ . In contrast, 35% of samples (7/20) from *Cfr*<sup>-/-</sup> *hCFTR*-G542X mice treated with 5 mg/kg of gentamicin yielded cAMP-stimulated short-circuit currents, resulting in an average current of 0.82  $\mu\text{A}/\text{cm}^2$ . These data confirm our

previous results showing that a high dose of gentamicin restores a statistically significant increase ( $P$  value < 0.05) in cAMP-stimulated currents relative to untreated controls. They also demonstrate that the administration of a clinically relevant dose of gentamicin to *Cfr*<sup>-/-</sup> *hCFTR*-G542X mice can restore a statistically significant increase ( $P$  value < 0.05) in cAMP-stimulated chloride currents relative to untreated controls, consistent with a partial restoration of CFTR protein and activity. However, the *hCFTR* activity observed in mice treated with the low dose of gentamicin was twofold less than the activity observed in mice treated with the higher gentamicin concentration.

We found that treatment of *Cfr*<sup>-/-</sup> *hCFTR*-G542X mice with 170 mg/kg of amikacin once daily for 2–3 weeks yielded a short-circuit response in 75% of intestinal tissues assayed (9/12), resulting in an average current of 1.77  $\mu\text{A}/\text{cm}^2$ . Treatment of these mice with 15 mg/kg of amikacin resulted in a cAMP-stimulated short-circuit response in 40% of intestinal samples (6/15), resulting in an average current of 1.31  $\mu\text{A}/\text{cm}^2$ . These results indicate that both high and low doses of amikacin result in a statistically significant increase ( $P$  value < 0.05) in cAMP-stimulated chloride currents relative to untreated controls, consistent with an increase in *hCFTR* protein and function. Furthermore, the average short-circuit current was 60% higher in samples from mice treated with a low dose of amikacin than in mice treated with a low dose of gentamicin.

## Discussion

The results of this study show that amikacin, like gentamicin, can effectively suppress the *hCFTR*-G542X stop mutation in a transgenic CF mouse model. In fact, we found that the low dose of amikacin tested in this study appears to suppress the *hCFTR*-G542X mutation more effectively than the low dose of gentamicin. This finding could have significant implications for the therapeutic suppression of premature stop mutations that cause diseases like CF, as doses that produce peak serum levels similar to those used in this study can be used clinically with lesser chances of aminoglycoside toxicity that results in serious side effects such as kidney failure and hearing loss.

Aminoglycosides are taken into cells via megalin, a multiligand, endocytic receptor that is particularly abundant in the proximal tubules of the kidney and the hair cells of the inner ear [30]. Upon entering kidney cells, the positively charged nature of aminoglycoside molecules has been shown to promote their association with acidic phospholipids in the lysosomal membrane [30, 31]. These interactions reduce the activity of a number of enzymes, including several phospholipases. This loss of enzymatic activity, combined with the production of free radical species, is thought to ultimately cause tissue damage. Based on these findings, amikacin may have several advantages over gentamicin for the suppression of premature stop mutations. Several studies have shown that

amikacin is not as toxic as gentamicin [31–34]. Amikacin is also taken up less efficiently by kidney tubular cells, exhibits less binding to acidic phospholipids, and inhibits lysosomal phospholipases to a lesser extent [32, 34]. Because the use of aminoglycosides to treat genetic diseases caused by stop mutations would require long-term administration on a daily or weekly basis, the reduced toxicity afforded by amikacin may be highly significant.

The molecular basis for these differences in toxicity may be related to how they are produced. Amikacin is a semisynthetic aminoglycoside that exists as a single isoform. In contrast, gentamicin is a naturally derived aminoglycoside that consists of a mixture of three isoforms: gentamicin C1, C1a, and C2. A structural study of gentamicin bound to the eukaryotic decoding site suggested that gentamicin C1a is primarily responsible for the suppression of premature stop mutations [35, 36]. Studies investigating the predisposition of the different isoforms of gentamicin to induce nephrotoxicity and ototoxicity found that the C2 isoform of gentamicin is significantly more toxic than the C1 or C1a forms [37, 38]. In addition, the clearance and steady-state serum concentrations of the different isoforms were found to differ after administration [38, 39]. Because the abundance of the C1, C1a, and C2 isoforms of gentamicin also vary significantly between different manufactured lots, the ability of various gentamicin preparations to suppress premature stop mutations without inducing toxicity could vary significantly. Given that our current results indicate that amikacin also provides a greater level of readthrough at clinical doses, this aminoglycoside may represent a safer, more effective alternative for suppression therapy.

Other approaches may further reduce the toxicity associated with aminoglycoside use. A recent study found that a regimen of individualized pharmacokinetic dosing of gentamicin or amikacin, rather than fixed dosages, increased their antibacterial efficiency while reducing toxicity [40]. Because the propensity of aminoglycosides to associate with lysosomal membranes and generate free radicals correlates well with toxicity, other approaches to reduce these complications are possible. For example, the use of antioxidant compounds to reduce free radical levels has been proposed [41–44]. In addition, both poly-L-aspartate [45, 46] and daptomycin [47, 48] have been shown to reduce the ability of aminoglycosides to interact with the phospholipids in the lysosomal membrane and thus reduce aminoglycoside-induced toxicity in animal models.

Previous *in vitro* studies have shown that aminoglycoside-mediated suppression of stop mutations typically results in 3–20% readthrough levels [4, 5, 8, 9, 12]. Accordingly, the level of hCFTR activity restored in these experiments is expected to represent only a fraction of normal hCFTR activity. This partial restoration of activity could explain why we detected hCFTR function in only a fraction (35–75%) of treated samples, as restoration of hCFTR activity in the negative samples may have been below the lower limit of our functional assay. With this caveat in mind, our data indicate that the average cAMP-stimulated chloride current measured

in samples from *Cftr*<sup>-/-</sup> mice was 13% of normal for the low dose of gentamicin and 22% of normal for the low dose of amikacin (calculated by comparing the average current measured in samples from the *Cftr*<sup>-/-</sup> mice to the current measured in samples from the *Cftr*<sup>+/+</sup> mice treated with the same dose of each compound). Because estimates of the level of hCFTR activity required for a therapeutic benefit range from 5% [49] to 10–35% [50] of the normal, these results suggest that the level of suppression achieved by aminoglycoside-mediated suppression therapy may ultimately be sufficient to achieve a therapeutic benefit in CF patients. However, it should be noted that this study was carried out with a transgenic mouse model. The expression of the human *CFTR*-G542X cDNA in this animal will undoubtedly be different than the expression of endogenous mouse *Cftr* that contains a premature stop mutation. Consequently, a firm conclusion about the *in vivo* efficacy of aminoglycoside-mediated suppression of *CFTR* premature stop mutations cannot be made until a better mouse model is obtained.

In our study, we found that the administration of amikacin at doses that produced serum levels within the clinically accepted range could suppress the *hCFTR*-G542X mutation and restore functional hCFTR protein. While gentamicin was also found to suppress the *hCFTR*-G542X mutation when administered in doses that produced serum levels in the accepted clinical range, the levels of suppression appeared to be significantly less than those observed with amikacin. The use of the administration protocol validated here in conjunction with other approaches to reduce their toxicity should further enhance the potential of these compounds for the suppression of premature stop mutations that cause genetic diseases.

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