Pharmacological Suppression of Premature Stop Mutations that Cause Genetic Diseases

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Abstract: Aminoglycoside antibiotics have long been used as antibacterial agents due to their ability to inhibit bacterial translation. However, aminoglycosides also stimulate translation errors in mammalian cells. Aminoglycosides bind to a pocket formed in a domain of the ribosomal RNA (rRNA) of the small ribosomal subunit that constitutes the decoding site in both prokaryotes and eukaryotes. Normally, accurate base pairing takes place between each successive codon and its cognate aminoacyl-tRNA within this region of the ribosome. When aminoglycosides bind to the decoding site, a conformational change decreases discrimination between cognate and near-cognate tRNAs, leading to errors in the decoding process. The ability of aminoglycosides to bind to the decoding site and induce translational misreading in eukaryotic cells is less efficient than in prokaryotic cells due to subtle differences in the sequence of the decoding site rRNA. The observation that aminoglycosides induce low levels of misreading in eukaryotic cells has inspired many investigations to determine whether aminoglycosides can suppress nonsense mutations that cause human diseases. Disease models in which aminoglycosides have been shown to efficiently suppress nonsense mutations include cystic fibrosis, Duchenne muscular dystrophy, Hurler syndrome, infantile neuronal lipfuscinosis, cystinosis, x-linked nephrogenic insipidus, spinal muscular atrophy, and cancer. However, if aminoglycosides are to be used clinically for suppression therapy, their efficacy must be improved and their toxicity reduced. The co-administration of other compounds that reduce aminoglycoside toxicity or the development of new compounds that suppress stop mutations may allow the realization of suppression therapy as a clinical treatment to suppress disease-causing stop mutations.

A. AMINOGLYCOSIDES SUPPRESS STOP CODONS IN PROKARYOTES AND EUKARYOTES

Discovery of Aminoglycoside-Mediated Suppression of Translation Termination in Prokaryotes

Aminoglycoside antibiotics are used primarily to treat infections caused by aerobic Gram-negative bacteria, where they exert their bactericidal effect by inhibiting protein synthesis. A large number of distinct compounds in the aminoglycoside family have been developed. The most common aminoglycosides in use today share a related structure comprised of a central 2-deoxystreptamine ring surrounded by adjacent amino sugars in either a 4,6-linkage (kanamycin, gentamicin, tobramycin, amikacin and G418) or a 4,5-linkage (paromomycin and neomycin). Streptomycin, another well-known aminoglycoside, contains a streptamine ring with a glycoside attached at position 4 (Fig. (**1**)).

While aminoglycosides inhibit bacterial protein synthesis when present at appropriate concentrations, exposure to lower, sub-lethal levels of some of these compounds induced the mis-incorporation of the wrong amino acid at a sense codon, or the insertion of an amino acid at a stop codon (resulting in translational readthrough) [Davies *et al*., 1964; Friedman and Weinstein, 1964; Lederberg *et al*., 1964]. This effect was termed phenotypic suppression, which refers to the suppression of a mutation without the occurrence of a secondary suppressor mutation. These results led to the conclusion that these compounds somehow reduced the fidelity of the decoding process during translation in prokaryotes.

Aminoglycosides Also Suppress Stop Codons in Eukaryotes

Since aminoglycosides were developed as antibiotics, research into their function was initially limited to how they altered translational fidelity in bacteria. However, it was later shown that a subset of aminoglycoside antibiotics could also induce misreading during eukaryotic protein synthesis. This was demonstrated using *in vitro* translation extracts derived from the ciliate *Tetrahymena thermophila* [Palmer and Wilhelm, 1978], wheat germ [Wilhelm *et al*., 1978b], and human cells [Wilhelm *et al*., 1978a]. Subsequently, it was demonstrated that aminoglycosides could also suppress stop mutations in growing yeast cells [Palmer *et al*., 1979; Singh *et al*., 1979] and in cultured mammalian cells [Burke and Mogg, 1985]. These results suggested that the mechanism that maintains translational fidelity is at least partially conserved between prokaryotes and eukaryotes.

B. MECHANISM OF AMINOGLYCOSIDE-INDUCED SUPPRESSION

The 70S ribosome of *E. coli* is made up of the small (30S) and large (50S) subunits. The observation that mutations conferring resistance to streptomycin are mediated by changes in the 30S ribosomal subunit [Cox *et al*., 1964; Davies, 1964] indicated that both a key component of translational fidelity and the target of aminoglycoside action was located on the same subunit previously shown to bind the mRNA molecule [Okamoto and Takanami, 1963]. Since that time, our understanding of the mechanism of

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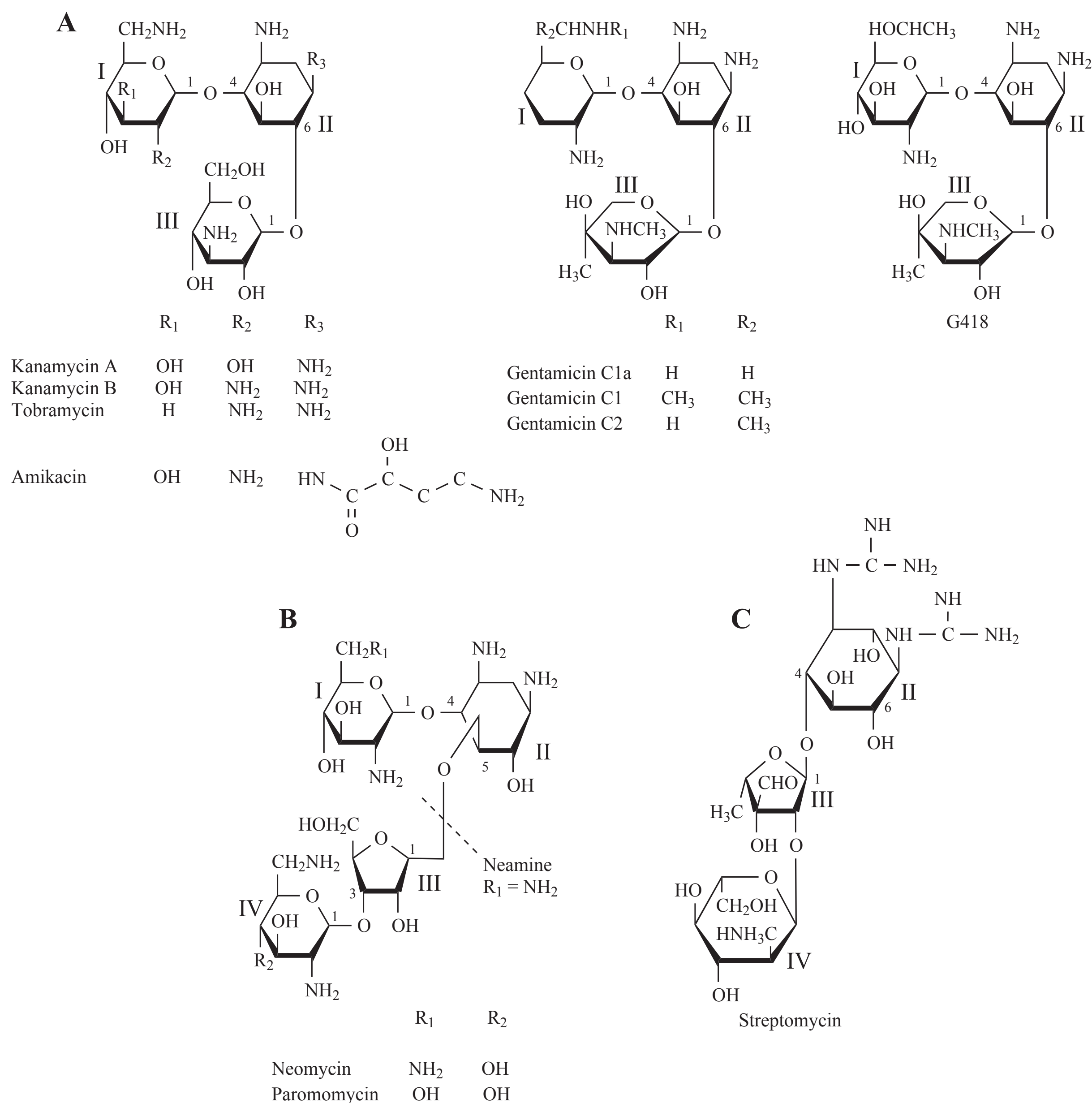

Fig. (1). Common aminoglycoside structures. A) Common aminoglycosides with a 2-deoxystreptamine core (labeled ring II) and attached aminosugars (labeled rings I and III) in a 4,6-linkage. B) Common aminoglycosides with a 2-deoxystreptamine core (labeled ring II) and attached aminosugars (labeled rings I, III and IV) in a 4,5-linkage. C) Streptomycin contains a streptamine ring (labeled ring II for consistency with the other structures) and attached aminosugars (labeled rings III and IV) in a 4-linkage.

aminoglycoside-induced fidelity errors has increased tremendously.

Identification of the Ribosomal Binding Site for Aminoglycosides

Early studies utilized chemical protection assays to identify the ribosomal site for aminoglycoside binding. For example, Moazed and Noller [Moazed and Noller, 1986] used chemical footprinting with the modifying agent dimethylsulfate (DMS) to identify changes in the protection pattern of rRNA that occurs when the bacterial 70S ribosome (or 30S ribosomal subunit) is treated with various aminoglycosides. The assumption in this approach was that the appearance of protected residues upon aminoglycoside addition would indicate their binding sites. The data suggested that neomycin, gentamicin, paromomycin, and kanamycin all bind strongly to residues A1408 and G1494 of 16S rRNA in intact 70S ribosomes as well as the isolated 30S subunit (Fig. (**2**)). This finding suggested that the aminoglycosides contacted these rRNA residues when bound to the intact bacterial ribosome. These rRNA residues were predicted to lie on either side of a portion of helix 44 termed the decoding region, based on their proposed role in mRNA decoding during translation.

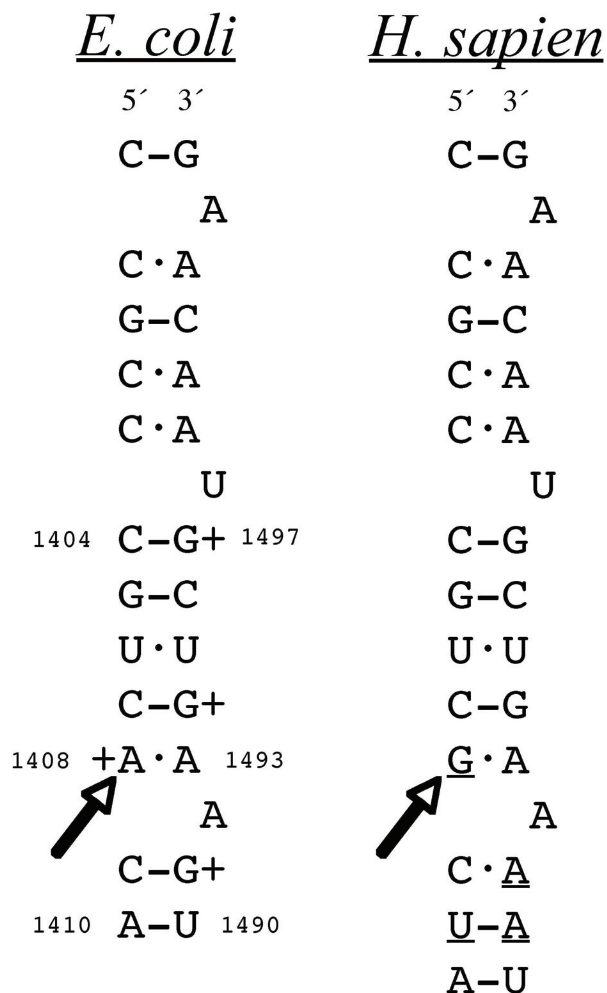

Fig. (2). Comparison of the small subunit rRNA decoding sites of the prokaryote *E. coli* and the eukaryote *H. sapien* (humans). Nucleotides not conserved between the two structures are underlined in the eukaryotic (*H. sapien*) structure. The positions of DMS footprints caused by aminoglycosides binding are indicated on the *E. coli* structure by (+) symbols. The invariant A residue (prokaryotes) and corresponding G residue (eukaryotes) are indicated by arrows.

A subsequent study found that the aminoglycosides neomycin and paromomycin could bind a small synthetic RNA designed to mimic the predicted structure of the bacterial decoding region in a manner that also protected residues A1408 and G1494 from chemical footprinting with DMS [Purohit and Stern, 1994]. This footprinting pattern was remarkably similar to the results obtained with intact 70S bacterial ribosomes, indicating that this model rRNA representing a small portion of the overall 16S rRNA structure could interact with these compounds at similar concentrations as those required to bind intact ribosomes. Residues G1491 and G1497 of this synthetic RNA were also protected to a lesser extent. The use of these synthetic decoding site RNAs subsequently allowed structural studies to be carried out that revealed the structure of the bacterial decoding site with and without various aminoglycosides bound [Fourmy *et al*., 1996; Fourmy *et al*., 1998a; Fourmy *et al*., 1998b; Recht *et al*., 1996; Vicens and Westhof, 2001; Yoshizawa *et al*., 1998]. It was found that aminoglycosides bound in a pocket formed in the major groove of helix 44 by the non-canonical A1408-A1493 base pair followed by the unpaired A1492 (Fig. (**2**)). These studies confirmed that members of both the kanamycin and neomycin classes of aminoglycosides contacted specific residues in the decoding region when bound to the bacterial 16S rRNA.

The Molecular Basis for Aminoglycoside-Induced Misreading

Structural studies have also examined the functional consequences of aminoglycoside binding to synthetic bacterial decoding site RNAs [Fourmy *et al*., 1998b; Vicens and Westhof, 2001] and the intact prokaryotic 30S ribosomal subunit [Carter *et al*., 2000]. It was found that paromomycin binding (particularly the contacts formed by rings I and II) induces the bases of decoding site residues A1492 and A1493 to flip out of the helix. Since these rings are generally conserved among the clinically relevant aminoglycosides, this conformational change is probably a common effect of aminoglycoside binding.

The high degree of accuracy during the translation process has long been an enigma, since the hydrogen bonding potential of codon-anticodon interactions alone are not sufficient to explain the high level of accuracy observed (estimated to be on the order of 99.9%). However, the conformational change of A1492 and A1493 provided important new insights into the mechanism of ribosomal decoding. In the flipped-out conformation, the bases point directly into the A site where they are positioned in a way that allows them to interact with the minor groove of the codon-anticodon helix. This novel interaction has been proposed to be the mechanism by which the decoding region monitors the proper codon-anticodon interaction by hydrogen bonding to 2′ hydroxyl groups on both sides of the codon-anticodon helix [Carter *et al*., 2000]. When combined with contacts involving other rRNA residues around the A site, these interactions are thought to provide a sensitive test for any distortions that could arise when an incorrect codon-anticodon pairing occurs. Remarkably, when aminoglycosides bind to the decoding site, the energetic cost of binding cognate tRNAs (where all three bases of the anticodon are complementary to the codon) and near-cognate tRNAs (where only two out of three bases of the anticodon are complementary to the codon) is reduced. This increases the affinity of both cognate and near-cognate tRNAs for the A site, thus increasing the chances of accepting an incorrect aminoacyl-tRNA during the selection process. As a consequence of this effect, the frequency of translational misreading is increased and the translation process becomes more error-prone.

The Prokaryotic Bias for Aminoglycoside Action

The fact that aminoglycoside antibiotics inhibit translation by prokaryotic ribosomes at concentrations 10-15 times lower than those required to inhibit eukaryotic protein synthesis allows them to be used therapeutically to treat bacterial infections [Wilhelm *et al*., 1978b]. As such, there must be some unique features in how aminoglycosides bind

to ribosomes from bacteria and humans. As shown in Fig. (**2**), there is a significant level of homology between the prokaryotic and eukaryotic decoding sites. Once the sequences of the small subunit rRNA from various prokaryotic and eukaryotic species became available, it was noted that bacterial 16S rRNAs contain an A residue at position 1408 (*E. coli* numbering), while the corresponding position in the eukaryotic decoding site is a G residue [Van de Peer *et al*., 1994]. This observation led to experiments to test the importance of this residue in determining the efficiency of aminoglycoside binding.

Using a model oligonucleotide that mimicked the proposed structure of the *E. coli* decoding site, it was shown that various sequence variants resulted in weaker binding of paromomycin to the oligonucleotide [Fourmy *et al*., 1996; Recht *et al*., 1996]. In particular, base changes known to confer aminoglycoside resistance in 16S rRNA were shown to result in weaker paromomycin binding. More importantly, it was found that some variants with nucleotides changed to the corresponding residues found in the eukaryotic decoding site showed a reduced level of aminoglycoside binding. This finding strongly suggested that these subtle sequence differences are responsible for the prokaryotic specificity of aminoglycosides. Further support for this model was obtained when it was shown that substitution of the bacterial A1408 residue previously implicated in aminoglycoside binding to the corresponding G residue found in eukaryotes allowed *E. coli* cells to grow in the presence of several hundred-fold higher concentrations of certain aminoglycosides than normal. Chemical footprinting experiments showed that the increased resistance was accompanied by weaker aminoglycoside binding [Recht *et al*., 1999]. These results confirmed residue A1408 (the base pairing partner of A1493) as a major determinant of aminoglycoside affinity, and is at least partially responsible for the specificity of aminoglycoside action in prokaryotes.

The Sequence Context Surrounding a Stop Codon Influences the Efficiency of Translation Termination

Many aspects of the overall process of translation termination are still poorly understood. For example, the three different stop codons (UAG, UAA, and UGA) vary in their efficiency to signal termination. In addition, the context surrounding the stop codons can have a surprisingly large effect on the efficiency of translation termination. In bacterial systems, it has been shown that the context both 3′ and 5′ of the stop codon plays an important role in determining the efficiency of the termination process [Bossi and Ruth, 1980; Miller and Albertini, 1983; Mottagui-Tabar *et al*., 1994].

Context-dependent effects on termination efficiency have also been shown in various eukaryotic systems. One of the important developments that allowed the characterization of differences in the efficiency of translation termination was the development of a series of sensitive readthrough reporter systems [Bonetti *et al*., 1995; Firoozan *et al*., 1991; Grentzmann *et al*., 1998; Keeling *et al*., 2004; Stahl *et al*., 1995]. These systems allowed the frequency of stop codon suppression to be followed by one of several sensitive enzymatic assays. Using such systems, it was shown that both the upstream and downstream context surrounding stop codons in eukaryotic systems can play an important role in determining the efficiency of translation termination [Bonetti *et al*., 1995; Brown *et al*., 1990; McCaughan *et al*., 1995; Namy *et al*., 2001]. In particular, the nucleotide directly 3′ of the stop codon exerts what is frequently the strongest influence on the efficiency of translation termination (other than the identity of the stop codon itself).

Eukaryotic cells contain two translation termination factors. The eRF1 protein is a class I release factor that is thought to interact directly with each of the three stop codons when they are located in the ribosomal A site [Frolova *et al*., 1994]. The eRF3 protein is a class II release factor that possesses GTPase activity and facilitates the function of eRF1 [Frolova *et al*., 1996; Stansfield *et al*., 1995; Zhouravleva *et al*., 1995]. It is not yet clear how the surrounding sequence context influences the efficiency of translation termination, but it is possible that eRF1 recognizes an extended termination signal. Interestingly, it was recently shown that the recognition of so-called "tetranucleotide termination signals" (containing the stop codon and the next 3′ base) was influenced by mutations in the eRF3 gene [Salas-Marco and Bedwell, 2004]. Thus, while eRF1 may directly contact the stop codon, eRF3 may also modulate the termination process through its interaction with eRF1 in a manner that facilitates the decoding of a subset of tetranucleotide termination signals. Recent studies using various reporter systems have also shown that the ability of aminoglycosides to suppress stop codons is context dependent [Howard *et al*., 2000; Keeling and Bedwell, 2002; Manuvakhova *et al*., 2000].

C. USE OF AMINOGLYCOSIDES TO SUPPRESS STOP MUTATIONS THAT CAUSE HUMAN DISEASES

Suppression Therapy to Treat Cystic Fibrosis

Cystic fibrosis (CF) is a common and lethal autosomal recessive disorder that is caused by dysfunction of the cystic fibrosis conductance regulator (CFTR) protein. The CFTR protein is a cAMP-activated chloride channel [Riordan *et al*., 1989]. Loss of CFTR activity results in abnormal chloride and sodium transport across the apical membrane of epithelial cells in the lung airways, pancreas, intestine, and the male reproductive system. The main cause of morbidity in CF patients is severe and chronic respiratory infections that result in respiratory failure and early death. The most common mutation, found in approximately two-thirds of CF patients, is the *CFTR*-ΔF508 mutation [Consortium, 1994]. The ΔF508 mutation, a three nucleotide deletion that results in the loss of a single phenylalanine residue, leads to the mislocalization and rapid turnover of the CFTR protein. Premature stop mutations represent approximately 5% of the total mutations found in CF patients worldwide. However, this number is much higher in certain CF populations, such as the Ashkenazi Jewish population, where 64% of the mutant alleles contain a premature stop mutation [Shoshani *et al*., 1992]. *CFTR* nonsense mutations produce little or no CFTR protein and also cause a severe reduction in steady-state *CFTR* mRNA levels due to the process of nonsense-mediated mRNA decay (NMD) [Hamosh *et al*., 1992;

Hamosh *et al*., 1991]. NMD is a quality-control mechanism found in all eukaryotic cells that actively eliminates mRNA molecules that contain a premature stop mutation [Holbrook *et al*., 2004]. It is thought that NMD evolved to prevent the expression of potentially harmful, truncated polypeptides that arise from errors in mRNA synthesis or splicing.

The ability of aminoglycosides to suppress disease-causing premature stop mutations was first tested using *CFTR* nonsense mutations [Howard *et al*., 1996]. Human *CFTR* cDNA constructs containing the G542X or R553X mutations (both having the TGAG tetranucleotide termination signal) were expressed in HeLa cells, which do not express endogenous CFTR. When these cells were grown in the presence of the aminoglycoside G418, full-length CFTR protein could be detected as a function of the aminoglycoside concentration in a dose-dependent manner. In addition, cAMP-activated CFTR chloride channel activity was also detected in G418-treated cells, demonstrating that the CFTR protein produced by stop codon suppression was functional. These studies were further expanded using a human bronchial epithelial cell line in which the *CFTR* nonsense mutation was expressed from the nuclear genome [Bedwell *et al*., 1997]. The IB3-1 cell line carries the W1282X nonsense mutation on one allele (with the TGAA tetranucleotide), and the ΔF508 mutation on the other allele [Zeitlin *et al*., 1991]. In the presence of G418 or gentamicin, activation of cAMP-dependent whole-cell chloride currents was restored. CFTR protein localization to the apical cell surface was also detected using immunofluorescence techniques after aminoglycoside treatment. Importantly, it was also shown that *CFTR* mRNA expressed from the W1282X allele was stabilized due to suppression of the nonsense mutation by aminoglycosides, suggesting that suppression of the premature stop mutation may be able to counteract the NMD process. A later study showed that CFTR function could also be restored in human biliary epithelial cells after gentamicin treatment as measured by whole cell recordings of CFTR chloride channel activity [Zsembery *et al*., 2002].

Aminoglycosides have also been shown to suppress a *CFTR* premature stop mutation in a transgenic CF mouse model [Du *et al*., 2002]. In this study, a human *CFTR* transgene that carried the G542X mutation (with the TGAG tetranucleotide) was stably expressed in a *Cftr-/-* mouse under the control of the rat fatty acid binding protein promoter (*FABP*). This mouse was found to have a pathophysiology common to that of other *Cftr-/-* mice, where intestinal blockage caused death in approximately 80% of mice shortly after weaning [Davidson and Rolfe, 2001; Hasty *et al*., 1995; Ratcliff *et al*., 1993; Snouwaert *et al*., 1992]. One week prior to weaning, a treatment regimen was begun in which *Cftr-/- FABP-hCFTR*-G542X mice were administered 34 mg/kg of gentamicin or tobramycin daily *via* subcutaneous injection. Immunofluorescence studies using intestinal tissues revealed the appearance of human CFTR protein at the apical surface of the intestinal submucosal glands of these mice following gentamicin or tobramycin treatment (Fig. (**3**)). Using short circuit current measurements, a restoration of cAMP-activated CFTR chloride channel activity was also observed in the intestinal tissue of gentamicin-treated mice, and to a lesser extent, of tobramycin-treated mice. The ability of gentamicin to suppress the *CFTR-G542X* mutation much more efficiently than tobramycin was confirmed using an *in vitro* translation system.

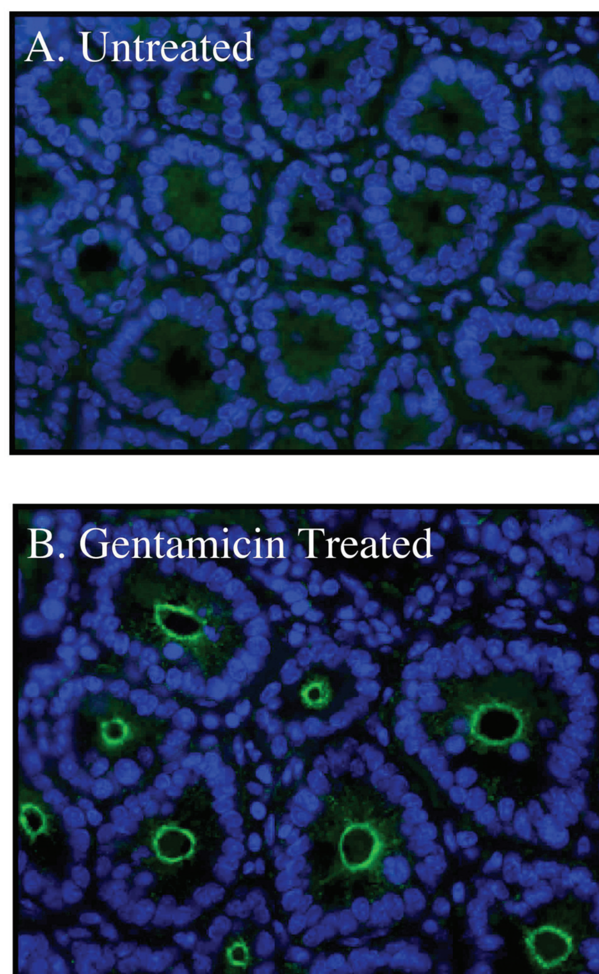

Fig. (3). Immunofluorescence staining of mouse intestinal tissues from *Cftr-/-* mice carrying a human *CFTR*-G542X transgene using CFTR-specific antibodies. A) Immunofluorescence of intestinal ducts from untreated *Cftr-/-* mice carrying the *CFTR*-G542X transgene. B) Immunofluorescence of tissue from the same mice showing CFTR at the apical surface of intestinal ducts following once-daily subcutaneous injections with 34 mg/kg gentamicin for 2 weeks. The blue staining is nuclei; the green staining is CFTR protein.

Although a partial restoration of CFTR expression and function was observed in *Cftr-/- FABP-hCFTR*-G542X mice after aminoglycoside treatment, no significant increase in survival was found. Several reasons could account for this negative result. First, the cell-type specificity of CFTR expression in intestinal tissues influences the survival of CF mice [Manson *et al*., 1997; Rozmahel *et al*., 1997; Zhou *et al*., 1994]. Due to the random insertion of the *hCFTR-G542X* transgene into the mouse genome and its expression from the heterologous *FABP* promoter, it was quite possible that an adequate level of *CFTR* expression was not present in the

appropriate cell types to increase the survival of these animals. Second, the human and murine CFTR proteins retain only 78% identity at the amino acid level, and it has been suggested that human CFTR may not be able to fully complement the multiple functions and signaling pathways in which murine CFTR participates [Zhou *et al*., 1994]. Finally, an adequate level of functional CFTR may not have been produced in the mouse intestinal tissues by aminoglycoside suppression of the G542X mutation. It has been estimated that at least five percent of the wild-type CFTR level is needed to improve survival in CF mice [Dorin *et al*., 1996]. Similarly, it was reported that a five percent level of CFTR protein is also sufficient to ameliorate the severity of lung disease in CF patients [Ramalho *et al*., 2002]. Clearly, more sophisticated mouse models than are currently available are needed to address these complex issues.

Three clinical trials have also been published in which CF patients that carry nonsense mutations were administered gentamicin and examined for the appearance of CFTR function. In the first study, nine subjects with *CFTR* premature stop mutations were administered intranasal gentamicin drops at a concentration of 3 mg/ml three times daily for a total of 14 days [Wilschanski *et al*., 2000]. Nasal potential difference measurements were carried out to monitor CFTR function before and after gentamicin treatment. A statistically significant increase in chloride transport was observed following aminoglycoside treatment. In another study, 2.5 mg/kg gentamicin was administered intravenously three times daily for seven days to two experimental groups: one group of five CF subjects that carried nonsense mutations, and a second group with five other CF subjects that carried other *CFTR* mutations as controls [Clancy *et al*., 2001]. Although no significant difference in chloride conductance was observed using sweat chloride testing, nasal potential difference assays detected a significant increase in cAMP-stimulated chloride transport in the CF subjects with nonsense mutations after gentamicin treatment. The five control CF subjects did not respond to aminoglycoside treatment, indicating that the therapeutic affect associated with aminoglycoside treatment was specific for subjects with nonsense mutations. More recently, a double-blind, placebo-controlled, crossover trial was conducted in which 3 mg/ml gentamicin droplets were administered to CF subjects three times daily for 14 days [Wilschanski *et al*., 2003]. Nasal potential difference measurements indicated a restoration of CFTR activity in 90% of the gentamicin-treated subjects with nonsense mutations. In addition, CFTR protein could be detected at the apical membrane of epithelial cells obtained from gentamicin-treated CF subjects that carried nonsense mutations. When taken together, the results of these clinical trials suggest that aminoglycosides may be of clinical significance for the treatment of CF caused by *CFTR* nonsense mutations.

Suppression of Stop Mutations to Treat Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked lethal, degenerative disorder that is caused by an absence of the protein dystrophin. Dystrophin is a large (427 kDa) sarcolemmal structural protein that coordinates multiple protein interactions that link the extracellular matrix to the cytoplasmic cytoskeleton. This disorder affects 1 in 3,500 male births. Approximately 15% of all DMD cases are due to nonsense mutations [Mendell and Dietz, 2001]. Small population studies suggest that approximately 40% to 50% of DMD patients carry a nonsense mutation in some populations [Prior *et al*., 1995].

The initial studies designed to examine the ability of aminoglycosides to suppress nonsense mutations in the dystrophin gene utilized a Duchenne muscular dystrophy mouse model referred to as the *mdx* mouse. This mouse carries a spontaneous Q995X nonsense mutation in the dystrophin gene (with a TAAA tetranucleotide), and the pathophysiology in the *mdx* mouse is very similar to human DMD disease. Treatment of the *mdx* mice with 34 mg/kg gentamicin *via* once daily subcutaneous injections restored 20% of normal dystrophin protein levels (as well as dystrophin-interacting proteins) as determined by immunofluorescence and western blot analysis [Barton-Davis *et al*., 1999]. In addition, functional muscle contraction assays demonstrated that gentamicin treatment also prevented the accumulation of contractile injury that occurred in the *mdx* mouse as a function of physical exercise. A significant reduction in serum creatine kinase activity was also observed in the gentamicin-treated *mdx* mice. Another group found that the same gentamicin treatment regimen also led to a significant recovery of vascular function and cell density in the *mdx* mouse model [Loufrani *et al*., 2004]. However, a third group reported an inability to duplicate the results in the initial Barton-Davis study using the *mdx* mouse [Dunant *et al*., 2003]. They were unable to detect dystrophin protein in muscle tissue from gentamicin-treated animals by immunoblotting methods in experiments where a detection limit of 3% of normal dystrophin levels was demonstrated. In the same mouse model, Barton-Davis *et al*. reported a restoration of 20% of wild-type dystrophin levels after aminoglycoside treatment using a similar immunoblotting method [Barton-Davis *et al*., 1999]. Notably, other *in vitro* studies of termination suppression at various tetranucleotide termination signals have shown that the TAAA tetranucleotide termination signal present at the nonsense mutation found in the *mdx* mouse is the most resistant termination signal to aminoglycoside-mediated suppression [Howard *et al*., 2000; Keeling and Bedwell, 2002; Manuvakhova *et al*., 2000]. Therefore, a restoration of 20% of wild-type dystrophin protein in the Barton-Davis study might be an overestimate. However, it is difficult to compare the quantitation between the two *mdx* mouse studies directly, since the immunoblotting conditions and antibodies used to quantitate the dystrophin levels were different. Another possible explanation for the contrasting results in these studies could be differences in the composition of the gentamicin administered to the mice, since commercial gentamicin preparations are known to contain varying amounts of the C1, C1a, and C2 gentamicin species.

Mixed results have also been reported in small clinical trials in which DMD subjects that carry nonsense mutations were administered aminoglycosides. In one study, four patients that carried nonsense mutations in the dystrophin gene were intravenously administered 7.5 mg/kg once a day

[Wagner *et al*., 2001]. No increase in dystrophin levels was observed in any subjects, nor was physical improvement observed using muscular strength tests. However, serum creatine levels were significantly reduced in the patients post-treatment. In a more recent study, four DMD patients with nonsense mutations were administered 6.0-7.5 mg/kg gentamicin by an intravenous route once daily [Politano *et al*., 2003]. Muscle biopsy revealed a partial restoration of dystrophin levels by immunoblot and immunofluorescence in three of the patients that carried the TGA stop codon (TGAC tetranucleotide). The fourth patient that carried a TAA nonsense mutation (TAAA tetranucleotide) did not show a restoration of dystrophin. These results are consistent with other studies showing that the TGAC termination signal is much more susceptible to aminoglycoside suppression than the TAAA termination signal [Howard *et al*., 2000; Keeling and Bedwell, 2002; Manuvakhova *et al*., 2000].

Other Disease Models Used to Test Stop Codon Suppression Therapy

The ability of aminoglycosides to suppress a number of other disease-causing nonsense mutations has also been investigated. Gentamicin has been shown to suppress a number of different stop mutations that cause lysosomal storage diseases that result in progressive and lethal neuro-degeneration. In one study, it was shown that gentamicin can suppress the common nonsense mutations W402X (TAGG tetranucleotide) and Q70X (TAGC tetranucleotide) in the α-L-iduronidase (*IDUA*) gene [Keeling *et al*., 2001]. These mutations cause Hurler syndrome, the most severe form of Mucopolysaccharidosis I (MPS I). Gentamicin treatment suppressed these mutations in human primary Hurler fibroblasts, resulting in a restoration of 3% of normal α-L-iduronidase activity. In agreement with previous observations [Aronovich *et al*., 1996; Ashton *et al*., 1992], this level of activity was found to alleviate the Hurler disease phenotype, as measured by a reduced level of glycosaminoglycan storage products and a normalization of lysosomal morphology. A more recent study showed that a variety of nonsense mutations in the *IDUA* gene can be suppressed by gentamicin in mammalian cells [Hein *et al*., 2004]. Another study found that the common R208X stop mutation (TGAT tetranucleotide) in the *CLN2* gene, which codes for tripeptidyl-peptidase 1 (TPP-I) and is associated with infantile neuronal ceroid lipofuscinosis, can be suppressed in human fibroblasts grown in the presence of gentamicin [Sleat *et al*., 2001]. It was shown that up to 7% of normal TPP-I levels could be detected in gentamicin-treated cells. Similarly, it was shown that the W138X nonsense mutation (TGAT tetranucleotide) in the *CTNS* gene that causes cystinosis can be suppressed by gentamicin [Helip-Wooley *et al*., 2002]. Cystinosis fibroblasts that carried the W138X mutation showed both a restoration of cystinosin protein in the lysosomes by immunofluorescence as well as a reduced level of cystine storage in the lysosomes. These studies suggests that nonsense suppression therapy may be especially useful in the treatment of diseases caused by the lack of an enzyme in a biochemical pathway, since the restoration of even a very low amount of that enzyme may produce a significant phenotypic improvement. However, no clinical studies have yet been done to evaluate the ability of aminoglycosides to alleviate the diverse symptoms of patients with these diseases. For example, it has not been determined whether an adequate concentration of aminoglycosides can permeate the blood-brain barrier to counteract the mental deterioration associated with many lysosomal storage diseases.

Aminoglycosides have also been shown to suppress nonsense mutations that cause a number of other disorders. Schulz *et al*. showed that nonsense mutations in the V_2 vasopressin receptor gene (*AVPR2*) that are associated with X-linked nephrogenic diabetes insipidus (NDI) can be suppressed by gentamicin [Schulz *et al*., 2002]. An *AVPR2* plasmid construct that carried the W200X (TGAG tetranucleotide) or the R337X (TGAA tetranucleotide) NDI mutations could be suppressed in COS-7 cells when grown in the presence of gentamicin, as indicated by a robust AVPR2-dependent cAMP response and restoration of full-length AVPR2 protein. Another study showed that the W102X (TGAT tetranucleotide) mutation in the *SMN1* gene that causes autosomal recessive spinal muscular atrophy (SMA), a motor neuron disease, can be suppressed by gentamicin in cultured SMA fibroblasts [Sossi *et al*., 2001]. It was also demonstrated that nonsense mutations in the *P53* gene that are associated with tumor formation could be suppressed by aminoglycosides in a cell-free translation system and in mammalian cells [Keeling and Bedwell, 2002]. Similarly, Lai *et al*. found that aminoglycosides could suppress nonsense mutations in the *ATM* gene in human lymphoblastoid cell lines derived from ataxia telangiectasia patients [Lai *et al*., 2004]. After aminoglycoside treatment, the ATM protein could be detected by immunological methods, and the ability of cells to resist radiation-induced DNA damage was significantly improved. In contrast, another study showed less favorable results in the ability of gentamicin to suppress a nonsense mutation in the *RP2* gene that is associated with X-linked retinitis pigmentosa [Grayson *et al*., 2002]. After growing a human lympho-blastoid cell line that contained an R210X mutation (UGAG tetranucleotide) in the *RP2* gene in the presence of gentamicin, the RP2 protein could not be detected using immunoblotting methods capable of detecting approximately 0.4% of wild-type RP2 protein levels. Control reporter constructs that contained the UGAG tetranucleotide termination signal were also analyzed in Chinese hamster ovary cells in the presence of gentamicin and indicated that the gentamicin used in that study was capable of suppressing the UGAG tetranucleotide termination to approximately one percent of the normal RP2 protein level. These results suggest that this UGAG stop signal in the *RP2* gene may not be significantly responsive to aminoglycoside-mediated suppression due to additional context effects. However, it is also possible that some lymphoblastoid cells lines may be more resistant to suppression due to other factors, such as poor gentamicin permeability across the plasma membrane, an unusual localization of the RP2 protein, or reduced mRNA stability due to the NMD pathway.

D. FUTURE OBJECTIVES OF SUPPRESSION THERAPY

While the proof of concept for aminoglycoside-mediated suppression of premature stop mutations has been well-

documented by the many studies described in the previous section, there are still important questions that must be answered before aminoglycosides can be used as a viable, long-term strategy to suppress nonsense mutations in a clinical setting.

Improve Aminoglycoside Potency

The ability of aminoglycosides to suppress termination at nonsense mutations is substantially affected by the termination signal, the surrounding sequence context, and the structure of the aminoglycoside itself. Therefore, analysis of each nonsense mutation and its surrounding context with various aminoglycosides will be required to identify the aminoglycoside that would be most useful to suppress a particular mutation. Currently, a limited group of aminoglycosides that includes gentamicin, tobramycin, and amikacin is approved for internal administration in humans. More data is needed to confirm that *in vitro* suppression of a stop codon in its "natural" sequence context can be reliably correlated to *in vivo* suppression. A better understanding of the mechanism by which the surrounding sequence context affects the ability of aminoglycosides to suppress a termination codon is also needed.

Decrease Aminoglycoside Toxicity

The major limitation to the use of clinically approved aminoglycosides to treat bacterial infections or to suppress stop mutations is the fact that they frequently cause nephrotoxicity and ototoxicity in treated patients. Some aminoglycosides that are particularly effective for nonsense suppression (for example, G418) are considered too toxic for human use. Thus, only a limited number of aminoglycosides are currently available for use in the suppression of stop mutations in individuals with genetic diseases. Although the exact mechanism by which aminoglycosides cause toxicity is not fully understood, it appears that their toxicity is not due primarily to the ability of aminoglycosides to induce translational misreading, but rather to their charged nature.

The mechanism of aminoglycoside-induced toxicity appears to entail a complex series of steps [Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004]. Aminoglycosides are able to interact electrostatically with the negatively charged cell membrane and bind to megalin receptors that are especially numerous in the kidney proximal tubules and the inner ear. Megalin is a multi-ligand, endocytic receptor that is involved in the uptake of aminoglycosides [Nagai and Takano, 2004]. The role of megalin in renal aminoglycoside accumulation is strongly supported by a recent study showing that a mouse model carrying a knockout of the megalin gene does not accumulate aminoglycosides in the kidney [Schmitz *et al*., 2002]. Another study demonstrated that the administration of agonists that compete for aminoglycoside binding to megalin also results in a reduction in aminoglycoside uptake and toxicity [Watanabe *et al*., 2004]. These studies indicate that approaches aimed at reducing aminoglycoside uptake into effected cells by competition with megalin binding may reduce aminoglycoside-induced side effects.

Upon entering the cell, the positively charged nature of aminoglycosides allows their interaction with acidic phospholipids in the lysosomal membrane. This interaction alters the activity of a number of proteins and is thought to generate free radical species that leads to tissue damage. Many avenues of research have been pursued in an attempt to alleviate the toxicity associated with aminoglycosides. These have included the use of antioxidant compounds to reduce free radical levels [Kawamoto *et al*., 2004; Mazzon *et al*., 2001; Nakashima *et al*., 2000; Sener *et al*., 2002], and the use of poly-L-aspartate [Beauchamp *et al*., 1990; Gilbert *et al*., 1989] and daptomycin [Thibault *et al*., 1994; Thibault *et al*., 1995] to reduce the ability of aminoglycosides to interact with the phospholipids in the lysosomal membrane. In addition, altering the manner in which aminoglycosides are administered has been investigated as a means to reduce toxicity. The most common protocols used for the treatment of bacterial infections include either a once daily or a three-times daily administration of aminoglycosides [Bartal *et al*., 2003; Beauchamp and Labrecque, 2001]. However, unique protocols may need to be designed for the administration of aminoglycosides to suppress mutations that depend upon the type of nonsense mutation, the type of aminoglycoside administered, the route of administration, and the half-life of the restored protein. In addition, new administration methods may be developed to further reduce aminoglycoside toxicity. For example, it was recently demonstrated that gentamicin and amikacin encased in unilamellar liposomes exhibit a reduced rate of renal clearance that results in a substantial reduction in nephrotoxicity [Schiffelers *et al*., 2001].

Develop Alternative Drugs for Nonsense Suppression Therapy

Even though various approaches to reduce aminoglycoside toxicity have been investigated, few have been implemented into standard clinical use other than changes in the administration schedule. One study reported that it may be possible to separate elements of the aminoglycoside structure that induce toxicity from those that are required for an antibiotic effect [Maldague *et al*., 1984]. It may therefore be possible to discover or design compounds that will induce nonsense suppression in a manner similar to aminoglycosides without producing the toxic side effects associated with these compounds.

Negamycin is a dipeptide antibiotic that binds to the decoding site of the ribosome and alters translational accuracy in a manner similar to the aminoglycosides. It has been shown that negamycin can suppress nonsense mutations in the *mdx* mouse model for Duchenne muscular dystrophy [Arakawa *et al*., 2003]. It was also noted that negamycin is much less toxic than gentamicin. Investigation of the various translational components and their function may reveal completely new targets for the suppression of stop mutations such as other regions of the rRNA, ribosomal proteins, or the polypeptide chain release factors. Other possible therapeutic targets include factors involved in the NMD pathway that degrades mRNA transcripts containing nonsense mutations [Holbrook *et al*., 2004]. By stabilizing mRNAs that contain nonsense mutations, lower levels of nonsense suppression would be required to restore physiologically significant protein levels. Support for this therapeutic strategy has been obtained using cells from a patient with Ullrich's disease. In one study, the turnover of a

collagen VI α2 mRNA with a 26 nucleotide deletion (leading to an in-frame stop codon and the induction of NMD) was inhibited in cells treated with caffeine or wortmannin [Usuki *et al*., 2004]. These compounds have been shown to inhibit the kinase activity of hSMG-1 that is required for NMD function [Pal *et al*., 2001; Yamashita *et al*., 2001]. Stabilization of the collagen VI α2 mRNA did not correct the altered reading frame caused by the deletion, but restored the production of enough truncated collagen protein to partially restore extracellular matrix assembly (apparently, the deleted C-terminal amino acids did not eliminate collagen function). With greater understanding of the translation and mRNA turnover processes, single or combined treatments may be possible that restore protein expression by suppressing nonsense mutations, as well as by correcting the mRNA stability defects associated with NMD.

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